LIPID METABOLISM, LEARNING ABILITY AND POTENTIAL BIOMARKERS FOR ATHEROSCLEROSIS IN MONK PARROTS (*MYIOPSITTA MONACHUS*) FED N-3 FATTY ACIDS

A Dissertation

by

CHRISTINA A PETZINGER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Nutrition

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Approved by:

Co-Chairs of Committee,	John E. Bauer
	J. Jill Heatley
Committee Members,	Christopher A. Bailey
	Stephen B. Smith
Intercollegiate Faculty Head,	Rosemary L. Walzem

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ABSTRACT

Lipid Metabolism, Learning Ability and Potential Biomarkers for Atherosclerosis in Monk Parrots (*Myiopsitta monachus*) Fed N-3 Fatty Acids. (May 2012)

Christina A Petzinger, B.S., Berry College

Co-Chairs of Advisory Committee: Dr. John E. Bauer
Dr. J. Jill Heatley

Atherosclerosis, an inflammatory disease characterized by plaque formation in the arteries, commonly occurs in mammals, including humans, and some avian species. Polyunsaturated n-3 fatty acids have been shown to reduce known mammalian risk factors associated with the development of atherosclerosis in mammals. N-3 polyunsaturated fatty acids (PUFA) have also been linked to improving retinal, neurological, and brain development and functioning. In order to assess the effects of n-3 PUFA on potential risk factors for atherosclerosis in avian species and learning ability, a series of studies were conducted in Monk parrots: 1) alterations comparing a high linoleic acid diet with a high α-linolenic acid (ALA) diet on lipid metabolism, fatty acid conversions, and lipoproteins, 2) the dose response of ALA and comparison with a high docosahexaenoic acid (DHA) diet on lipid metabolism, fatty acid conversions, and markers of oxidation, 3) the effect of a high DHA diet on learning ability, and 4) assessment of growing energy requirement estimations to improve adult health.

Monk parrots were able to convert ALA to DHA and also retro-convert DHA/docosapentaenoic acid (DPA) to eicosapentaenoic acid (EPA). Feeding Monk parrots a high ALA diet resulted in a shift in the peak density of the high-density lipoproteins after 70 days. Decreased superoxide dismutase and increased malondialdehyde were observed by day 63 regardless of dietary n-3 PUFA levels or source. Higher plasma phospholipid DHA levels at day 28 were obtained when n-3 PUFA were provided in the diet as DHA rather than ALA (at equivalent amounts). Total plasma cholesterol, free cholesterol, esterified cholesterol, and triacylglycerol concentrations were not altered by increasing dietary n-3 PUFA. An effect of DHA on learning ability could not be concluded due to decreased power from adjusting for an age effect. Additionally, the growing energy needs for Monk parrots through day 23 after hatching were estimated and, unlike previous general equations, accounted for changes in growth energy requirements. These closer energy estimations that accounted for growth energy variations will hopefully prevent negative fluctuations in growth rate which were observed in the study and prevent obese fledgling and young adult birds.

In conclusion, Monk parrots are able to benefit from dietary n-3 PUFA provided as either ALA or DHA. Although, dietary DHA may provide more protection against the development of atherosclerosis due to its higher accumulation into plasma phospholipids and retro-conversion to EPA. However, caution should be used when feeding PUFA, as they increase oxidation in the body. While many risk factors for atherosclerosis have been determined in humans and other mammals, some of these do not appear to hold for Monk parrots and possibly other avian species prone to atherosclerosis.

DEDICATION

To my family and friends

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NOMENCLATURE

AA	Arachidonic Acid
ACC	Acclimation Diet
ALA	α-Linolenic Acid
BCA	Bicinchoninic Acid
BCS	Body Condition Score
CAT	Catalase
DHA	Docosahexaenoic Acid, Docosahexaenoic Acid Diet
DPA	Docosapentaenoic Acid
EC	Esterified Cholesterol
EOOC	Extinguish the Open One Cube Behavior
EPA	Eicosapentaenoic Acid
FAMEs	Fatty Acid Methyl Esters
FC	Free Cholesterol
FLX	Flaxseed Diet
HALA	High α-Linolenic Acid Diet
HDL	High-Density Lipoprotein
LA	Linoleic Acid
LALA	Low α-Linolenic Acid Diet
LDL	Low-Density Lipoprotein
LP-C	Lipoprotein Cholesterol

MALA	Medium α-Linolenic Acid Diet
MDA	Malondialdehyde
OOC	Open One Cube Learning Task
PUFA	Polyunsaturated Fatty Acid
ROOC	Relearn Open One Cube Learning Task
SCD	Stearoyl-CoA Desaturase
SFA	Saturated Fatty Acid
SOD	Superoxide Dismutase
SUN	Sunflower Seed Diet
TAG	Triacylglycerols
TC	Total Cholesterol
TCT	Three Cube Trial
TLC	Thin Layer Chromatography
VLDL	Very Low-Density Lipoprotein

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CHAPTER I

INTRODUCTION

Etiology of Atherosclerosis

Atherosclerosis begins with the formation of fatty streaks, which can eventually progress into fibrous plaques and complicated lesions. Plaques are composed of accumulations of lipids, cholesterol, foam cells, macrophages, and other leukocytes. Severe atherosclerosis results in plaques that disturb the vessel lumen while decreasing the diameter of the vessel core and increasing vessel wall thickness (1). Stenosis can occur in the vessel lumen due to circumferential arterial lesions. Mineralization of the artery can also occur in severe cases. Atherosclerosis is now known to be an inflammatory disease, resulting after long periods of chronic inflammation (1).

Atherosclerosis and Avian Species

Avian species affected by atherosclerosis consists of many orders including Anseriformes (waterfowl), Columbiformes (pigeons and doves), Galliformes (chickens and turkeys), and Psittaciformes (parrots) (2, 3). The most commonly affected Psittacine species are *Amazona aestiva* (Blue Fronted Amazon), *Psittacus erithacus* (African Grey parrot), *Myiopsitta monachus* (Monk parrot), *Cacatuidae spp*. (cockatoos), and macaws (4, 5). As an order, birds appear to be more susceptible to atherosclerosis than any mammal except humans (6). Atherosclerosis is an ongoing health concern in humans and antemortem risk factors for this disorder have been widely studied. However, the

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disorder seen in birds is primarily recognized on post-mortem examination. Avian plaques in chickens infected with herpesvirus (Marek's disease) closely resemble those that form in humans (7). Multiple retrospective studies at necropsy place the incidence of Psittaciforme atherosclerosis from 2% to 25% (2, 3, 5, 8). Most parrots are negatively affected by atherosclerosis by 2 to 13 years of age (3, 8). Atherosclerosis is probably greatly reducing the longevity of parrots in captivity. Monk parrots have been recorded to live at least 22 years in captivity, but based on observations from the Schubot Exotic Bird Health Center at Texas A&M University 5-10 year old birds commonly die from atherosclerosis (9).

Monk parrots originate from South America and have established numerous feral populations throughout the United States. They are slightly larger than parakeets, weighing approximately 100g. These parrots are commonly kept as pets due to their small size and good mimicking abilities, so the public has an invested interest in their health and improved longevity. Monk parrots are commonly referred to as Quaker parrots, Monk parakeets, and their scientific name, *Myiopsitta monachus*.

In Monk parrots, atherosclerosis occurs at a rate of ~10% based on a 10 year review of Schubot Exotic Bird Health Center (located at Texas A&M University) necropsy records, and is comparable to Psittaciformes in general (10). Factors predisposing birds to atherosclerosis remain unclear, but hyperlipidemia, endothelial inflammation, toxic substances, immune complex formation, species of bird, inappropriate long term diet (possibly high fat or cholesterol) and lack of exercise have been implicated (5). Risk factors for this disease in birds are poorly characterized. In

humans and other mammals, elevations of plasma cholesterol and both low and very low density lipoprotein cholesterol are positively associated with this disorder, while high density lipoprotein cholesterol elevations appear to be protective (11). Chronic postprandial lipemia has also been associated with the development of atherosclerosis (11, 12). Although caution should be taken with laying females because their plasma triacylglycerol concentrations have been observed to be elevated resulting in lipemic blood samples even after fasting. The lipemia due to lipid metabolism alterations during laying has not been linked to causing the same effects as the chronic postprandial lipemia that is a risk factor for atherosclerosis. Increased lipemia in females during laying has been observed in at least bobwhites (13) and Monk parrots (personal observation). Also, Monk parrots are frequently observed to have elevated plasma cholesterol concentrations compared to mammals, but the lipoprotein distribution of this lipid in the circulation and whether pro-atherogenic particles are increased under these conditions are poorly characterized to date. The most common clinical sign for birds with atherosclerosis is sudden death; however, a small percentage of birds with later stages of atherosclerosis display clinical signs consistent with circulatory problems (6). These clinical signs can include lethargy, dyspnea, fainting or sudden falling, and nervous symptoms due to blood loss in areas of the body (6). Once birds display these symptoms, it is often too late to prevent death (6), so it is important to identify and determine biological markers that can be used to help identify risk factors or even diagnose atherosclerosis while in the early stages.

Atherosclerosis and Oxidation

The formation of reactive oxygen species, free radicals, and lipid peroxides commonly occurs in animals. In excess, these compounds are toxic and can cause damage to cells in the body (14). Superoxide dismutase and catalase are two enzymes responsible for converting these toxic compounds into less or non-toxic compounds (14-16). Superoxide dismutase is a metalloenzyme responsible for converting the superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide. Hydrogen peroxide is then converted to water by catalase.

One current theory on the etiology of atherosclerosis is that low-density lipoproteins become trapped under the tunica intima in the artery (1). Once trapped, the cholesterol in the low-density lipoproteins can undergo oxidation and then be incorporated into macrophages, resulting in the production of lipid peroxides (1). Malondialdehyde is released when lipid peroxidation occurs and may thus be an indicator for the environment conducive to the development of atherosclerosis. In fact, malondialdehyde has been observed to be increased in humans with atherosclerosis (17, 18).

Atherosclerosis and n-3 Fatty Acids

Conversion of n-3 fatty acids in the body

Flaxseeds, camelina, chia seed, and walnuts contain large proportions of α-linolenic acid (18:3n-3, ALA). In contrast, linoleic acid (18:2n-6, LA), is found in substantial amounts in corn and sunflower seeds. Animals, unlike plants, do not have Δ12 or Δ15 desaturase enzymes, so ALA and LA cannot be synthesized de novo (19).

Due to these phenomena, animals must obtain ALA and LA from their diet. Once consumed, ALA and LA can be converted through a series of elongase and desaturase enzymes to longer chain derivatives (Figure 1). Eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are the most prominent long chain derivatives of ALA, while arachidonic acid (20:4n-6, AA) results from LA conversion.

The Δ6 desaturase is the first step in converting ALA and LA to their respective long chain derivatives. It is also the rate limiting step (19). In dogs, the Km for Δ6 desaturase is lower for ALA than LA. However, a much higher Vmax for Δ6 desaturase for ALA exists in both dogs and rats (20). Thus, once the Km for the Δ6 desaturase for ALA is met, ALA may be desaturated more rapidly than LA. Typical commercially available diets are much higher in LA than ALA, thus considerable conversion of LA to AA occurs despite the high Km and low Vmax the Δ6 desaturase has for this substrate.

The conversion rate of ALA to DHA in humans is less than 1% (21), so pre-formed DHA must be included in the diet in order to optimize its tissue accretion. It is noteworthy that, unlike humans, chickens can efficiently convert ALA into EPA and DHA (22). However, it is unknown if this same phenomenon occurs in parrot species.

Dietary n-3 fatty acids and atherosclerotic risk factors

In humans and other mammals, polyunsaturated n-3 fatty acids have been found to have beneficial effects on risk factors for atherosclerosis due in large part to a reduction of the inflammatory component seen in its etiology (23-25). For example, an ALA enriched diet fed to rats resulted in lowered plasma total cholesterol and phospholipid concentrations when compared to a high LA diet (26). However, it is

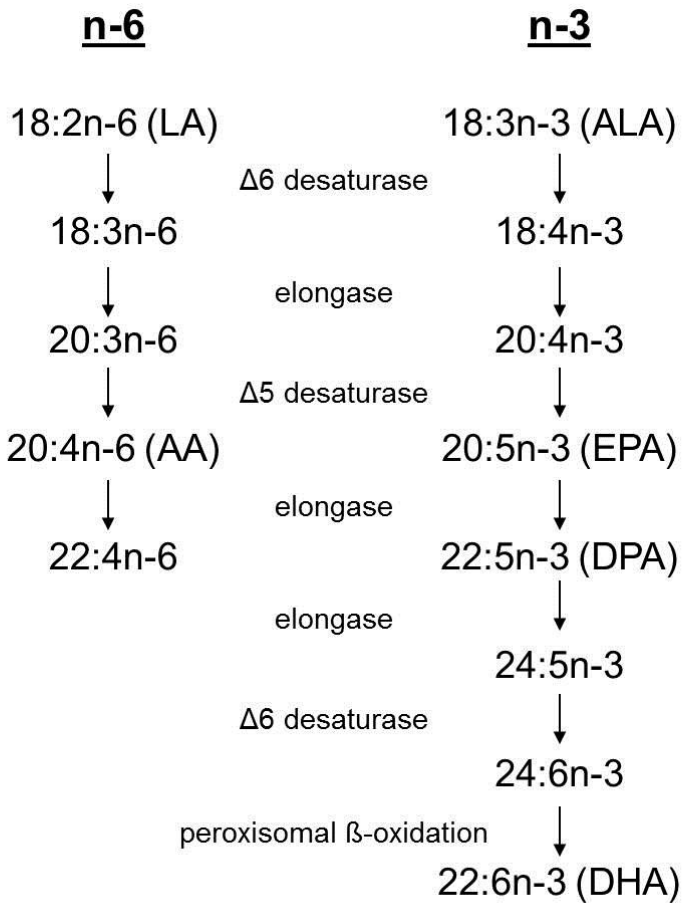

Figure 1. Conversion of α-linolenic acid and linoleic acid to their respective long-chain derivatives.

unknown whether ALA containing diets may positively affect traditional risk factors for atherosclerosis in avian species. However, a study in parrots found an inverse relationship between breast muscle and adipose ALA levels, which are indicative of dietary ALA, and atherosclerotic plaques found on necropsy (27). Feeding of n-3 fats do appear to be safe in birds because the addition of ALA using a 10% flaxseed containing diet showed no significant effect on feed intake of chickens, egg production, or liver

pathology and resulted in decreased liver fat (28). Long term feeding of a diet containing 10% flaxseed reduced the mortality rate in commercial laying hens (29).

Because of competition between LA and ALA for enzymatic conversion to longer chain derivatives that are precursors of inflammatory mediators, increased amounts of ALA may thus prevent the long-chain derivatives of LA, which are more pro-inflammatory, from being produced. This substrate competition for enzymatic conversion between n fatty acid families further suggests that the LA:ALA ratio, as well as the total amounts fed, are important dietary factors.

Increasing the amount of n-3 fatty acids relative to the n-6 fatty acids results in a shift in both plasma and platelet fatty acid profiles (30). Additionally, dietary polyunsaturated fatty acids become incorporated into cell membranes resulting in an increase in membrane fluidity while decreasing insulin resistance (31, 32). Dietary n-3 fatty acids have a more pronounced effect on decreasing inflammatory states in tissues due to EPA substituting for AA into cellular membranes and reduced conversion of AA to pro-inflammatory eicosanoids (32). The most beneficial health effects regarding atherosclerosis have been attributed to EPA and DHA, although some benefits may also result from ALA and other n-3 fatty acid intermediates (32). One possible reason for this is that both EPA and DHA are now believed to be important mediators of inflammation both directly and via eicosanoids (33).

The majority of studies investigating atherosclerosis in birds have utilized turkeys, Japanese quails, chickens, and pigeons (13). The reason these studies utilized these species may be due to their being readily available as well as their economic

interest. However, parrots differ physiologically from these commonly studied avian species. Thus, it is of additional benefit to study atherosclerosis in parrots in order to determine any major similarities and differences in the development or treatment of atherosclerosis among the various species.

Atherosclerosis and Obesity: Implications for Avian Pets

Obesity, accumulation of excess body fat, causes an increase in the inflammatory state of the body through oxidative stress (34, 35). Thus, obesity may increase the risk of developing atherosclerosis. Childhood obesity in humans has been implicated as a major risk factor for developing atherosclerosis as an adult due to the high number who remain obese as adults (36). This same phenomenon could potentially occur in parrots.

There is considerable variation in the current hand-rearing practices for pet birds. Most breeders utilize successful methods from years of practice that likely focus on survival rate rather than optimal health. When there are gaps in knowledge, it is common to look to information on poultry species; however, these birds have been studied in order to maximize growth rate for meat production, which is undesirable for pets and conservation purposes as it could lead to obesity later on.

The Protective Effect of High-Density Lipoproteins

High-density lipoproteins (HDL) have long been considered to have a protective role against atherosclerotic risk. More recently, however, specific subfractions of HDL have been observed to affect the development of atherosclerosis to varying degrees. For example, an increase in HDL_{2b} has been associated with a reduced incidence of atherosclerosis in humans (37) while the combined HDL_3 fraction (HDL_{3a}, HDL_{3b}, and

HDL_{3c}) appeared to reduce the amount of low-density lipoprotein (LDL) oxidation in vitro (38). High-density lipoproteins are responsible for transporting cellular cholesterol back to the liver, with HDL_3 displaying greater efficiency than HDL_2 in this phenomenon (39). However, inflammation has been shown to impair reverse cholesterol transport by HDL in vivo using mice (40). Both avians and canines have HDL as their predominant lipoprotein fraction. This characteristic is believed to be one of the mechanisms that prevents the development of atherosclerosis in canines (41). However, by contrast, parrots nonetheless commonly develop this disorder. Thus, focusing on differences between the two species may provide further insights into the metabolic complexity of atherosclerosis and potentially help identify species specific biological risk factors for this disease.

Other Effects of Long Chain n-3 Fatty Acids: Roles of Docosahexaenoic Acid

Docosahexaenoic acid is a long-chain n-3 fatty acid that is incorporated into the retina, brain, and other neural tissues (42). Puppies fed high levels of DHA during gestation, suckling, and weaning had improved retina responses as determined via electroretinography (43). Moriguchi and Salem (44) showed that ALA deficient mice improved motor performance upon higher dietary inclusion of ALA. Brain tissue from these mice deficient in ALA and then supplemented, showed increased DHA along with decreased DPA n-6 fatty acid composition.

Preliminary Study

Most commercially available parrot feeds are either extruded pieces, seed mixes, or a combination. Pelleted diets are typically not commercially available because many

birds and their owners refuse to accept them (45-48). Therefore, a preliminary study was performed to determine whether Monk parrots would readily accept, consume, and be maintained on a pelleted, non-extruded feed. Current feeding practices at the Schubot Exotic Bird Health Center at Texas A&M University were also evaluated to better quantify the amounts of feed being provided to the Monk parrots in the colony due to the possibility that large excess amounts of feed were being provided, which would risk overfeeding and resultant obesity. Having such information would better enable future dietary studies to be designed and executed using these birds.

Four Monk Parrots, recently acquired by the Schubot Exotic Bird Health Center at Texas A&M University, were utilized for this study after a quarantine period. The birds were maintained in an outside aviary with a solid roof and mesh steel sides with a heat lamp supplied when the temperature fell below 4.4°C (40°F). A thermometer located in the same room as the Monk parrots recorded the low and high temperatures each day. They were housed in pairs in hanging galvanized steel cages measuring 0.61 m x 0.61 m x 0.61 m (2’ x 2’ x 2’). A basket, made from the same galvanized steel as the cage, was covered in a plastic sheet to catch any dropped feed and was hung underneath the cage. The birds were provided *ad libitum* water and feed in metal bowls that was changed daily. Adequate perches were provided along with sticks from tree branches without leaves for enrichment.

The birds were maintained on a diet of 100% Zupreem Fruitblend® (Shawnee, KS) for the first 7 days of the trial (their previous diet). The following two weeks consisted of gradually transitioning the Monk Parrots onto a diet containing a pelleted

broiler grower diet (courtesy of Dr. Chris A. Bailey, Poultry Science Department, Texas A&M University). The final 6 days utilized a diet composed of 25% Zupreem Fruitblend® and 75% pelleted broiler grower diet. Birds were provided 25-30 g of food per bird, equivalent to what had been provided prior to the study.

Feed intake was measured over days 0-6 (Feed period 1, November 9-15, 2009) and 20-26 (Feed period 2, November 30-December 5, 2009). Feed intake was assessed by weighing the amount of feed given and subtracting the amount of feed remaining in the feed dish the next day along with the amount of feed dropped. The amount of feed in the water bowl was estimated based on average as-is pellet size and weight (0.14 g for 1 piece of Zupreem Fruitblend® and 0.12 g for 1 piece of pelleted broiler grower diet). For feed period 2, feed types were separated after collection, so feed intake could be determined for each type of feed. The same person estimated the amount of feed in the water throughout the study for consistency. A crucible served as a desiccation standard for feed intake and aided in accounting for moisture alterations due to the environment. Briefly, a small amount of feed was weighed and placed in a crucible. The crucible was then placed in the same room as the Monk parrots, but in a location where the birds could not touch or interfere with it. The crucible was removed and weighed at the same time as the feed collected from the previous day's feeding. The amount of feed removed and feed dropped were modified using the information from the desiccation standard to correct for weight changes due to moisture content. The desiccation standard allowed for a more precise feed intake calculation.

Total feed consumption during feed period 1 averaged 10.4 g/bird/day. During feed period 2, total feed consumption averaged 13.4 g/ bird/day. The birds consumed, on average, a 50:50 (w/w) ratio of the two diets during the second intake trial period. However, the majority of offered Zupreem Fruitblend® was consumed each day (86.7% versus 27.6% pelleted diet), suggesting that it was favored over the pelleted broiler grower diet.

Increased consumption during the second feed intake period may possibly be due to a decrease in average ambient temperature. It is also possible that the type of diet offered (extruded, pelleted, etc.) affected the amount these birds consumed. Consuming the majority of a previously available feed item during diet transitions is not necessarily unusual as birds are believed to prefer feeds they are familiar with, in this case the Zupreem Fruitblend® (49).

Because the birds were housed in pairs, the amount consumed per bird was calculated to be approximately half that consumed by the pair. While not a precise measurement, this approach provided a reasonable estimate for determining the amount of feed required for later trials using additional numbers of birds and cages. Another limitation to measuring intake in this study is that some feed particles were too small to collect when dropped. Also, because some feed fell into the water dish, the number of pieces and weights of total feed in the water could only be estimated. Finally, the birds experienced changes in temperature and weather because the facility was not fully secured from the outside elements. While insects and spiders could access the cages and food, no evidence was ever seen of insects either in the feed or having been eaten by a

bird. A heat lamp was added to the cages on December 2, 2009 around 5:00pm and remained for the duration of the study. This modest increase in temperature may have lowered feed consumption slightly, as the parrots did not have to use as much energy for heat production. Temperatures reached near freezing on December 3-5, 2009. During this time, one of the Monk parrots was observed sneezing on December 2, 2009 and may have been a sign of illness, which can also affect feed intake.

In spite of its limitations, this preliminary trial achieved both of its objectives. An estimated feed consumption of 11.8 g/bird/day was therefore determined. Although excess feed (equivalent to the average feed intake) should be provided to ensure adequate amounts due to changes in feed consumption and messiness of the bird. During this study, one cage wasted an amount of feed equivalent to half of its intake on a daily basis. Second, the results demonstrated that Monk parrots will consume pelleted diets. Although the birds in this study started eating the pellets in 1.5 weeks, other birds may take longer to adjust. It is also useful to note that the birds successfully transitioned from a multi-colored diet consisting of orange, purple, green, and yellow feed particles to a tan/brown colored diet with relative ease. It is currently unknown what role color plays in the acceptance of a feed item by Monk parrots. It is of interest, however, that during the first feeding period (7 days), one of the cages had primarily yellow feed in the water bowl and predominantly purple pieces uneaten. This was not observed for the other cage in the study. It is unknown whether this observation has any significance regarding color preferences of Monk parrots at this time.

Objectives and Hypotheses

The overall objective of this dissertation was to evaluate the metabolic effects of dietary n-3 fatty acids and its implications in altering atherosclerotic risk factors and learning ability. The specific objectives were to 1) evaluate the ability of Monk parrots to convert ALA to EPA and DHA and whether retro-conversion back to EPA might occur, 2) study the effect of n-3 fatty acids on lipid metabolism and the oxidative state of the body, 3) investigate the existence of a metabolic saturation response to ALA, 4) examine the effect of DHA on learning ability, and 5) better understand estimated energy requirements for hand-rearing Monk parrots to potentially improve health as adults.

It was hypothesized that Monk parrots can convert ALA to EPA but not DHA, as is the case in most mammals, and that longer chain n-3 fatty acids can be retro-converted back to EPA. It was also hypothesized that lipid metabolism of Monk parrots is differently altered with diets enriched in n-3 fatty acids (ALA or DHA) versus those enriched in n-6 fatty acids, with n-3 fatty acids additionally decreasing the oxidative state of the body. It was hypothesized that a metabolic saturation response would occur at high dietary levels of ALA. Additionally, it was hypothesized that DHA would improve the learning ability of Monk parrots. Another hypothesis was that the energy requirements of Monk parrots during post-hatching development can be estimated by comparing their growth curves to those of parent-raised birds.

CHAPTER II

MONK PARROT DIETARY FATTY ACID STUDY – POLYUNSATURATED FATTY ACID CONVERSIONS

Introduction

The family of n-3 fatty acids, especially eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), have consistently been shown to reduce atherosclerotic risk factors in humans and other mammals (50, 51). However, conversion of α-linolenic acid (18:3n-3, ALA) to DHA is not very efficient (21), so most mammals must consume EPA and DHA for these benefits. In this study, the effects of ALA on plasma lipids, lipoprotein metabolism by electrophoresis and ultra-gradient centrifugation, and atherosclerotic risk factors known for mammals were investigated. The ability of Monk parrots (*Myiopsitta monachus*) to convert ALA to EPA and/or DHA was also evaluated. Flaxseed is a good source of ALA, however it has been reported to reduce feed intake and production in poultry, especially when inclusion levels exceeded 10% (52-54). Therefore, the effect of including 10% ground flaxseed, as a source of ALA, in the diet on feed intake, body weight, and body condition score was also assessed in addition to its metabolic effects on lipid metabolism in Monk parrots.

Materials and Methods

This study utilized 25 adult Monk parrots (*Myiopsitta monachus*) housed at the Schubot Exotic Bird Health Center at Texas A&M University. Each bird received a physical examination, including body weight and condition score assessment, gender

determination, and was permanently identified with a metal leg band prior to starting the study. Chemistry profiles (Vetscan with Avian/Reptilian Profile Plus; Abaxis, Union City, CA) and complete blood counts were also analyzed to assess the general health status of each bird. All birds were maintained in 0.61m x 1.22m x 1.83m (2' x 4' x 6') cages in pairs (except for two birds, due to the original odd number of birds available for the study and the subsequent death of a cage-mate during the acclimation period). Birds were housed in a covered aviary with two steel mesh walls, which provided protection from inclement weather. In the winter, heavy curtains were secured over the steel mesh walls and heat lamps were utilized when the temperature fell below 4.4°C (40°F). When the temperature rose above 32.2°C (90°F), a swamp cooler and large wall fans were used to cool the aviary. Birds were provided feed and water *ad libitum*.

Birds were transitioned to a pelleted, complete, and balanced acclimation (ACC) diet (corn-soybean based) for 5 weeks (-5 weeks) prior to feeding the study diets. The ACC diet consisted of corn, soybean meal, alfalfa, molasses, vitamin pre-mix, and mineral pre-mix (Table 1). The total acclimation period lasted from February 16, 2010 through March 22-24, 2010, due to staggered start dates. For the first six days of the acclimation period, the birds were fed 75% Zupreem Natural® and 25% ACC diet. The

Table 1. Diet ingredients and nutrient composition of the acclimation (ACC), sunflower seed (SUN), and flaxseed (FLX) diets.[1]

Nutrient	ACC	SUN	FLX
Corn, Yellow	66.3	56.3	56.3
Soybean Meal, Dehulled (48%)	18.4	18.4	18.4
Molasses, Dehydrated	2.0	2.0	2.0
Alfalfa, Meal (17%)	9.0	9.0	9.0
Sunflower Seeds, Ground	0.0	10.0	0.0
Flaxseeds, Ground	0.0	0.0	10.0
DL-Methionine (98%)	0.48	0.48	0.48
L-Lysine Hydrochloride	0.02	0.02	0.02
Limestone	1.3	1.3	1.3
Mono- + Dicalcium Phosphate	1.8	1.8	1.8
Sodium Chloride	0.43	0.43	0.43
Mineral Premix[2]	0.05	0.05	0.05
Vitamin Premix[3]	0.25	0.25	0.25
Moisture	12.8	9.7	10.2
Crude Protein	18.3	17.9	18.4
Crude Fiber	3.4	4.5	3.8
Ash	6.0	6.9	6.7
Fat	4.0	5.7	6.1

[1]Nutrient composition values are on an "as fed" basis and expressed as a percentage of the total diet.
[2]The TAMU mineral pre-mix contained 52.8 g/kg iron as ferrous sulfate, calcium carbonate, 136.4 g/kg manganese as manganous oxide, 110 g/kg zinc as zinc oxide, 8.8 g/kg copper as copper sulfate, and 2.2 g/kg iodine as calcium iodate, 200 mg/kg selenium, and 3.7 g/kg molybdenum.
[3]The TAMU vitamin pre-mix contained ground rice hulls, calcium carbonate, mineral oil, 1.4 g/kg vitamin A and 38.6 mg/kg vitamin D as vitamin A acetate in gelatin-sugar-starch beadlet (preserved with Ethoxyquin) and vitamin A acetate with vitamin D_3 supplement (preserved with Ethoxyquin), 10 g/kg vitamin E, 8.8 g/kg vitamin B_{12}, 2.6 g/kg riboflavin, 17.6 g/kg niacin, 4.4 g/kg pantothenic acid as calcium pantothenate, 198 g/kg choline as choline chloride, 589 mg/kg vitamin K as menadione sodium bisulfite complex, 440 mg/kg folic acid, 1.6 g/kg pyridoxine as pyridoxine hydrochloride, 792 mg/kg thiamin as thiamin mononitrate, 44 mg/kg biotin as d-biotin.

Table 2. Fatty acid composition of the acclimation (ACC), sunflower seed (SUN), and flaxseed (FLX) diets.[1]

	Fatty Acid	ACC	SUN	FLX
Saturated fatty acids				
	16:0	14.5	8.4	9.5
	18:0	2.6	4.0	4.3
	20:0	0.5	0.4	0.4
	22:0	0.2	0.7	0.3
	24:0	0.3	0.3	0.3
	Total SFA[2]	18.1	13.8	14.7
Monounsaturated fatty acids				
	16:1	1.8	0.3	0.4
	18:1n-9	31.2	31.3	23.6
	18:1n-7	1.7	0.7	1.0
	20:1	0.3	0.2	0.2
	Total MUFA[2]	35.1	32.6	25.3
n-6 Polyunsaturated fatty acids				
	18:2n-6 (LA)	43.5	51.4	26.3
n-3 Polyunsaturated fatty acids				
	18:3n-3 (ALA)	2.8	2.3	33.6
	Total PUFA[2]	46.3	53.6	59.8
	ALA:LA Ratio	15.5	22.5	0.8

[1]Values are expressed as a percentage of total fatty acids. Each diet sample was analyzed in triplicate.
[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

following 6 days consisted of the birds being fed 50% Zupreem Natural® and 50% ACC diet. The next 6 days of feeding was comprised of 25% Zupreem Natural® and 75% ACC diet. For the remainder of the acclimation period, birds were fed 100% ACC diet. At the beginning of the feed trial (day 0) the birds were randomly divided into two groups [flaxseed (FLX) group or sunflower seed (SUN) group] and fed their respective experimental diet, again as a complete and balanced pelleted feed. The experimental diets were formulated and prepared by substituting 10% whole ground FLX or 10% ground dehulled SUN for that portion of the ground corn in the ACC formula (Table 1). The amount of feed offered (30 g/bird) was determined to be sufficient from the preliminary study. The ACC diet contained 4.0% fat and a linoleic acid (18:2n-6, LA):ALA ratio of 15.5, the FLX diet had 6.1% fat and a LA:ALA ratio of 0.8, and the SUN diet had 5.7% fat and a LA:ALA ratio of 22.5 (Table 2). Due to the death of one bird assigned to the SUN group from causes unrelated to the study on day 0, 13 birds in the FLX group and 11 birds in the SUN group completed the feeding trial.

Feed intake

Feed intake was measured on a daily basis. All measurements were taken using a Sartorius BP4100 scale (Bradford, MA). Feed intake was assessed by weighing the amount of feed offered and subtracting any amounts of feed remaining in the feed dish the next day along with the amount of feed dropped. To catch dropped feed, a wire mesh basket was hung from the cage and held a solid black plastic polyethylene tray slightly larger than the dimensions of the cage (Drs. Foster and Smith, Rhinelander, WI). In this way, feed particles that might be dropped through the cage would land on the tray and

could be brushed into pre-weighed crucibles using a 2" chip brush (Economy; Home Depot, Atlanta, GA). The amount of feed that dropped into the water bowl was estimated based on average as-is pellet size and weight, as follows. The average weight for a piece of Zupreem Natural® was 0.04 g, the ACC diet was 0.18 g, the SUN diet was 0.19 g, and the FLX diet was 0.20 g. A crucible served as a desiccation standard for feed intake and aided in accounting for moisture alterations due to the environment. Briefly, a small amount of feed was weighed and placed in a crucible. The crucible was then placed in the same room as the Monk parrots, but in a location where the birds could not touch or interfere with it. The crucible was removed and weighed at the same time as the feed collected from the previous day's feeding. The feed removed and feed dropped amounts were corrected using the information from the desiccation standard to account for weight changes due to moisture content (Appendix A). The desiccation standard allowed for a more precise feed intake calculation.

Body weight and body condition score

Body weights were measured on blood sampling days (-5 weeks, day 0, 7, 14, 28, 42, 56, and 70). Each bird was individually placed into a small perforated aluminum metal basket with lid after blood samples were collected. The basket was then placed on a tared scale (OD-ES011; Office Depot, Boca Raton, FL). Bodyweights were recorded to the nearest gram.

Body condition score (BCS) was also assessed on blood sampling days. Body condition was determined by palpation of pectoral muscles, subcutaneous fat, and the keel bone of the bird while being gently restrained manually. The BCS scale ranged

from one to five, with one being emaciated and five being obese (Figure 2). A BCS of one corresponded to a sharp V-shape that was concave with little to no pectoral muscle and fat being felt. A score of two was assigned when a smooth, flat V-shape was felt. A score of three was associated with a rounded or elliptical V-shape. A BCS of four corresponded to a semi-circle shape where the ridge of the keel could barely be felt. A score of five was assigned when pectoral muscles and fat stores bulged over each side of the keel making an M-shape. The same person was assigned to measure body condition score at each sampling period to reduce bias.

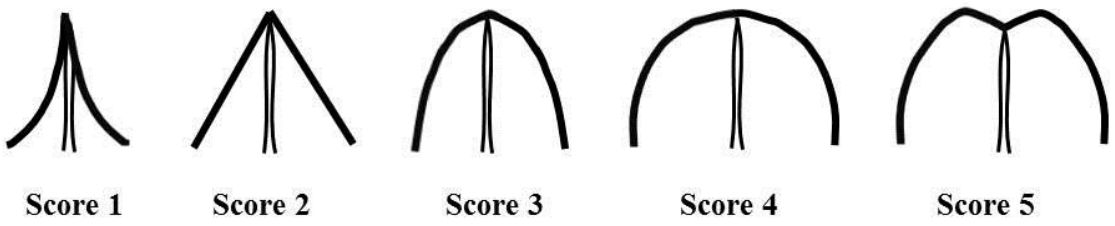

Figure 2. Body condition scores as visualized when palpated.
The top point and lines in the middle of each diagram represent the keel of the Monk parrot. A score of 1 corresponds to emaciation and a score of 5 to obesity.

Blood collection

Birds were caught using a net and/or towel and were manually restrained during the blood collection process. Blood samples (1 cc maximum) were obtained from the right jugular vein and placed into lithium heparin microtainers after withholding feed for

3 hours at -5 weeks, and days 0, 7, 14, 28, 42, 56, and 70. Feed was normally removed prior to sunrise on blood sampling days.

Plasma phospholipid fatty acid profiles

Plasma lipid extraction

Total lipids from Monk parrot plasma samples and diets were extracted following a modified protocol of Folch and colleagues (55, 56). Two hundred and fifty μL of plasma sample was added to a 12 mL teflon-lined screw-capped test tube. Then 4.5 μL of chloroform:methanol (2:1, v/v) with 0.2% glacial acetic acid was added to the 12 mL tube. The tubes were shaken for 20 minutes (Model S-500; Kraft Apparatus, Inc., Mineola, NY). Two mL of distilled water was added to the tube and mixed for another 10 minutes. The tubes were then spun in an Allegra X-15R centrifuge at 2,800 rpm (1,825 x g) for 15 minutes (Beckman Coulter, Brea, CA). The chloroform subnatant, which contained the extracted lipids, was then transferred to a clean 12 mL teflon-lined screw-capped test tube. An addition of 5 mL chloroform:methanol:distilled water (3:48:47, v/v/v) was pipetted into the new tube and placed on the shaker for 10 minutes. The tubes were next centrifuged at 2,800 rpm (1,825 x g) for 15 minutes. The chloroform layer (subnatant) was transferred to another clean 12 mL teflon-lined screw-capped test tube. A stream of nitrogen was passed over the surface of the chloroform solution. Lipid extract samples were then either stored at -20ºC less than a week or proceeded onto thin layer chromatography (TLC).

Thin layer chromatography (TLC)

If frozen, lipid extract samples were removed from the freezer and allowed to thaw at ambient temperature. All samples were evaporated to dryness under nitrogen. Then 200 µL of chloroform was added to each tube. A TLC tank lined with filter paper was filled with 2cm of chloroform:methanol (2:1, v/v), 2 cm from the bottom of the tank. Silica gel G coated plates (20x20cm, 250 µm thickness) were prewashed and activated in an oven at 110ºC for 1 hour. A separate TLC tank lined with filter paper was filled with hexane:diethyl ether:acetic acid (80:20:1, v/v/v), 2 cm from the tank bottom, and allowed to equilibrate for 1 hour. The activated plates were then loaded with 100 µL of each sample or standard (18-5-A, Nu-Check Prep, Inc., Elysian, MN). The 18-5-A standard consisted of equal parts (20% of each, weight basis) cholesterol, cholesterol oleate, triolein, oleic acid, and lecithin. Plates were developed until 1-2 cm from the top. The phospholipid fraction was then scraped into a clean screw-capped 12 mL test tube. The plate was next briefly visualized in an Iodine vapor chamber and the phospholipid spot was circled. Each circled fraction was scraped into a clean screw-capped test tube following complete disappearance of the yellow Iodine coloration. Two mL of 4% H_2SO_4 in methanol was added to each test tube. Nitrogen was streamed over the surface. Test tubes were then either stored at -20ºC less than a week or methylated.

Methylation of plasma samples

If samples were frozen, they were removed from freezer and allowed to thaw at ambient temperature. Samples were mixed on a vortex mixer and placed in a 90ºC water bath (Model 1235, VWR Scientific Products, Cornelius, OR) for 1 hour. Samples were

removed and cooled to room temperature. Three mL of hexane was added to each sample and mixed. Samples were centrifuged at 2,800 rpm (1,825 x g) for 15 minutes. The supernatant (hexane and fatty acid methyl esters, FAMEs) was transferred to a clean screw-capped test tube. Nitrogen was streamed over the sample. Samples were then stored at -20ºC less than a month or analyzed immediately for FAMEs on via gas chromatography.

Gas chromatography

For both the plasma and diet samples, 20 µL FAMEs in hexane were placed in a gas chromatography vial insert. The sample was placed on a Hewlett Packard 5890 Series Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA) autosampler. A FAMEWAX^{TM} fused silica capillary column (30 m long, 0.25 µm thick, and .32 mm internal diameter, Restek, Bellefonte, PA) had 2 µL sample injected into it. Helium was the carrier gas with an initial velocity at 28.3 cm/second and a flow of 1.25 mL/minute. The column was attached to a flame ionization detector. The initial oven temperature of 145ºC was held for 8 minutes. The temperature then increased at a rate of 1.7ºC/minute up to 220ºC. The temperature was then held for 10 minutes and began increasing again at a rate of 8ºC/minute to a final temperature of 250ºC. This temperature was maintained for 15 minutes to eliminate any debris from the column prior to running the next sample. Some plasma samples from day 70 were run at different settings due to shortening of the column. These samples had an initial temperature of 130ºC which was maintained for 10 minutes. The temperature then increased to 220ºC by 1.5ºC/minute. The temperature was held at 220ºC for 10 minutes prior to increasing by 8ºC/minute up to 250ºC. This final

temperature was maintained for 15 minutes. The accompanying software (ChemStation B.03.01, Agilent Technologies, Santa Clara, CA) created chromatograms from the detections. Retention times of a FAMEs standard (#68-B, plus 17:0, 18:1n7, 18:3n6, 20:5n3, 22:4n6, 22:5n3, Nu-Check Prep, Inc. Elysian, MN) was utilized to determine individual fatty acid peaks on the chromatogram.

Plasma total cholesterol, free cholesterol, esterified cholesterol, and triacylglycerol concentrations

Total cholesterol (TC), free cholesterol (FC), and triacylglycerol (TAG) concentrations were determined by colorimetric endpoint enzymatic assays according to Wright-Rodgers and colleagues (57). Esterified cholesterol (EC) concentration was determined by subtracting FC concentration from TC concentration.

Triacylglycerol and cholesterol determination

Colorimetric endpoint enzymatic assays were utilized to determine plasma TAG, TC, and FC concentrations. Six µL of plasma sample and 200 µL of the appropriate reagent (described below) were added to wells of a microplate. Standards (0, 50, 100, 200, 300, 500 mg/dL for TAG and 0, 50, 100, 200, 300, 400 mg/dL for TC and FC) were included on each microplate with distilled water serving as the 0 mg/dL standard. Standards and samples were tested in triplicate. Microplates were mixed, incubated at 37ºC for 20 minutes, and then equilibrated to room temperature for 20 minutes. A microplate reader (Molecular Devices Corporation, Sunnyvale, CA) and its accompanying software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA) measured the absorbance of each well at 490 nm.

Triacylglycerol reagent

Triglycerides GPO Reagent (Siemens, Tarrytown, NY) was a ready to use solution of 2.5 mmol/L ATP, 2.5 mmol/L Mg acetate, 0.8 mmol/L 4-Aminoantipyrine, 1.0 mmol/L 3,5-dichloro-2-hydroxybenzene sulfonate, >3000 U/L glycerol phosphate oxidase, >100 U/L glycerol kinase, >2000 U/L lipoprotein lipase, >300 U/L peroxidase, and 53 mmol/L buffer for a final pH of 7.0. Once combined with the plasma sample, the lipoprotein lipase hydrolyzes TAG into glycerol and non-esterified fatty acids. Glycerol was converted to glycerol-3-phosphate in the presence of glycerol kinase catalyst. Glycerol-3-phosphate was then oxidized in the presence of the glycerol phosphate oxidase catalyst to produce dihydroxyacetone phosphate and hydrogen peroxide. Hydrogen peroxide reacted with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzene sulfonate in the presence of a peroxidase catalyst to produce quinoneimine dye. Quinoneimine dye is red and proportional to the TAG concentration of the sample when absorbance is read at 490 nm.

Cholesterol reagents

The procedures for the enzymatic cholesterol reagents were described by Warnick (58). A 50 mmol/L PIPES buffer (pH6.9) was prepared by combining 17.30 g disodium salt (Sigma-Aldrich, St. Louis, MO), 1.292 g sodium cholate (Sigma-Aldrich, St. Louis, MO), and 1 mL Triton X-100 (Sigma-Aldrich, St. Louis, MO). One mol/L hydrochloric acid was used to adjust the PIPES buffer pH to 6.9 while warmed to 37ºC. Cholesterol reagent A consisted of adding 0.102 g 4-aminoantipyrine and 1.492 g potassium chloride (Sigma-Aldrich, St. Louis, MO) to 100 mL PIPES buffer. The

addition of 0.08 g 2-hydroxy-3,5-dichlorobenzene sulfonic acid (Sigma-Aldrich, St. Louis, MO) to 100 mL PIPES buffer constituted cholesterol reagent B. Cholesterol reagent C is comprised of cholesterol reagent A and B (1:1, v/v), 10 U/mL horseradish peroxidase, 0.5 U/mL cholesterol esterase, and 0.5 U/mL cholesterol oxidase (Sigma-Aldrich, St. Louis, MO). Cholesterol reagent C' had identical composition to cholesterol reagent C, but without cholesterol esterase. Cholesterol reagent C was used to determine TC concentration while cholesterol reagent C' determined FC concentration.

For cholesterol reagent C, addition to the sample resulted in cholesterol esters forming cholesterol and non-esterified fatty acids in the presence of cholesterol esterase catalyst. Cholesterol oxidase next catalyzed the oxidation of cholesterol to produce cholest-4-en-3-one and hydrogen peroxide. The hydrogen peroxide then reacted with 4-aminoantipyrine and 2-hydroxy-3,5-dichlorobenzene sulfonic acid with the horseradish peroxidase as catalyst to produce quinoneimine dye. Cholesterol reagent C' resulted in only free cholesterol undergoing oxidation in the presence of cholesterol oxidase. The remaining reaction steps proceeded as described above.

Plasma lipoproteins

Lipoproteins were fractionated via agarose gel electrophoresis as well as by a modified density gradient ultracentrifugation method (59).

Lipoprotein determination via electrophoresis

Lipoprotein fractions consisting of portomicron, β (low-density lipoprotein, LDL), pre-β (very low-density lipoprotein, VLDL), and α (high-density lipoprotein, HDL) were determined by electrophoresis via 1% agarose gel (TITAN GEL Lipoprotein

Electrophoresis System, Helena Laboratories, Beaumont, TX). The procedure comprised of the company provided instructions with minor modifications. The gel application area was blotted with Titan Gel Blotter A and 2 μL of plasma sample were applied. The samples were given 7 minutes to diffuse into the agarose. Meanwhile, 25 mL of TITAN GEL Lipoprotein Buffer, a barbital-sodium barbital buffer with thimerosal and 0.1% sodium azide as preservatives, was added to each inner section of the TITAN GEL Chamber. The gel was properly placed in the chamber and electrophoresed at 90 volts for 40 minutes. The gel was removed from chamber and dried for 40 minutes at 70ºC. The dried gel was immersed in a solution of 5 mL distilled water and 25 mL working solution for 2.5 minutes for visualization of the lipoprotein bands. The working solution consisted of 0.05 g Fat Red 7B dissolved into 200 mL of methanol. The gel was then destained by submersion in methanol-distilled water (1:1, v/v) for 20 seconds. Stain fixation was achieved by placing the gel in 2% glycerol in distilled water for 20 seconds then dried overnight at room temperature. Quantitative evaluation of lipoprotein bands was achieved by scanning at 525 nm with a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) in conjunction with its software (Quantity One, Bio-Rad, Hercules, CA). The lipoprotein distributions, as percentages of total lipoproteins, were transformed to lipoprotein-cholesterol (LP-C) data using total plasma cholesterol values.

Density gradient ultracentrifugation

A modified density gradient ultracentrifugation method (59) was used to determine the lipoprotein fraction distribution in plasma of the two experimental groups. Plasma samples and NBD C_6-ceramide were removed from the freezer and thawed at

room temperature. Samples and NBD C_6-ceramide were mixed. Forty µL 1 mg/mL NBD C_6-ceramide was added to a 1.5 mL eppendorf tube. Next, 1254 µL 0.18 M NaBiY gradient solution was added to the eppendorf tube. Six µL sample was added to the eppendorf tube. The tubes were then inverted 10 times and mixed for 10 seconds using a vortex mixer. UC tubes were first inspected for smoothness and 1150 µL of the solution was transferred from the eppendorf tube into the UC tube. UC tubes were placed in the centrifuge (Optima TLX Personal Benchtop Ultracentrifuge, Beckman Coulter, Inc., Brea, CA) and equilibrated at 5ºC for 30 minutes to allow ample time for staining. The samples were then centrifuged at 120,000 rpm (511,000 x g) at 5ºC for 6 hours. Approximately 200µL hexane was then layered into the UC tube to remove meniscus from the tube for imaging. All tubes were wiped with a Kimwipe (Kimberly-Clark Professional, Roswell, GA) prior to imaging to ensure no condensation was on the tube. Source filter was set at BG12 blue filter while the emission filter was set at OG515 yellow longpass filter. A calibration tube marked at 1mL increments was filled with distilled water and imaged using MicroFire® Monochrome Microscope Digital CCD Camera (1600x1200 pixel; Optronics®, Goleta, CA). The imaging settings for the calibration tube were a target intensity of 30%, exposure of 53.3 ms, and a gain of 1.0000 taken as a monochrome image. Sample images were taken at 18 ms. Following imaging, the image was translated into pixels using OriginPro 7 (OriginLab™ Corporation, Northampton, MA). Data was analyzed between 180-190 pixels. Integrated intensities were then calculated using Excel (Microsoft Corporation, Redmond, WA).

Diet fatty acid composition

Diet lipid extraction

Ten g of diet was randomly chosen and ground (coffee grinder; Mr. Coffee, Boca Raton, FL). An 800 mg subsample of the homogenized diet was transferred into a 50 mL round bottom test tube. Twenty mL of chloroform:methanol (2:1, v/v) and 0.2% glacial acetic acid were added to the subsample and mixed using a vortex mixer for 30 seconds. Nitrogen gas was streamed over the sample surface and samples were stored in a 6ºC refrigerator overnight. Following refrigeration, samples were shaken (Model S-500, Kraft Apparatus, Inc., Mineola, NY) for 30 minutes. Five mL of distilled water was added to each tube and the tubes were again shaken for 15 minutes. Samples were then centrifuged Allegra® X-15R Centrifuge, Beckman Coulter, Brea, CA) at 3,000 rpm (2,095 x g) for 15 minutes. The chloroform layer was transferred to a clean 50 mL round bottom test tube and set aside. Ten mL of chloroform:methanol (2:1, v/v) was added to the original test tube and shaken for 20 minutes. Two mL of distilled water was added to the tube and shaken an additional 10 minutes. The sample was then centrifuged at 3,000 rpm (2,095 x g) for 10 minutes. The bottom chloroform layer was added to the previously separated chloroform layer. Fifteen mL of chloroform:methanol:distilled water (3:48:47, v/v/v) was added to the test tube containing the pooled chloroform layers. The tubes were shaken for 10 minutes and centrifuged at 3,000 rpm (2,095 x g) for 10 minutes. The bottom chloroform layer was pipetted through a glass wool containing pipette into a clean screw-capped test tube. The sample was taken to dryness

by nitrogen gas. One mL of hexane was added to the sample. Samples were then methylated.

Trans-methylation of diet lipid extracts

Seventy five μL of the diet lipid extract was transferred into a clean screw-capped test tube and dried under nitrogen gas in order to prepare fatty acid methyl esters (FAMEs) for gas chromatographic analysis. Then, 2 mL of 4% H_2SO_4 in methanol was added to each tube and caps were secured. Samples were mixed for 30 seconds and placed in a 90ºC water bath for 1 hour. After the samples had cooled to room temperature, 3 mL hexane was added to each tube. Samples were then mixed for an additional 30 seconds and centrifuged at 3,000 rpm (2,095 x g) for 15 minutes. The top layer was transferred to a clean screw-capped test tube and nitrogen gas was streamed over the surface. Samples were then either stored at -20ºC or analyzed by gas chromatography.

Gas chromatography

For both the plasma and diet samples, 20 μL FAMEs in hexane were placed in a gas chromatography vial insert. The sample was placed on a Hewlett Packard 5890 Series Gas Chromatograph (Hewlett Packard Co., Palo Alto, CA) autosampler. A FAMEWAX^{TM} fused silica capillary column (30 m long, 0.25 μm thick, and .32 mm internal diameter, Restek, Bellefonte, PA) had 2 μL sample injected into it. Helium was the carrier gas with an initial velocity at 28.3 cm/second and a flow of 1.25 mL/minute. The column was attached to a flame ionization detector. The initial oven temperature of 155ºC was held for 8 minutes. The temperature was then increased at a rate of

1.5ºC/minute up to 220ºC. The temperature was then held for 15 minutes and increased again at a rate of 8ºC/minute to a final temperature of 250ºC. This temperature was maintained for 15 minutes to eliminate any debris from the column prior to running the next sample. The accompanying software (ChemStation B.03.01, Agilent Technologies, Santa Clara, CA) created chromatograms from the detections. Retention times of a FAMEs standard (#68-B, plus 17:0, 18:1n7, 18:3n6, 20:5n3, 22:4n6, 22:5n3, Nu-Check Prep, Inc. Elysian, MN) was utilized to determine individual fatty acid peaks on the chromatogram.

Diet fat content

Gravimetric determination

Samples were taken to dryness under nitrogen gas and redissolved in 1 mL of chloroform. A metal planchet was weighed to 5 decimal points (Ohaus Analytical Plus, Ohaus Corporation, Florham Park, NJ). Next, 100 µL sample was pipetted into the middle of the planchet. The planchet was then placed in a desiccator overnight to allow for solvent evaporation. The planchet was then reweighed to 5 decimal places. The following equation was used to determine the percent fat in the feed sample:

[(planchet with evaporated sample weight – planchet only weight) x 10] / weight of dry sample extracted x 100 = % fat in feed sample

Statistical analyses

Data were analyzed for normality using the Shapiro-Wilks test. Normally distributed data were then analyzed by repeated measures ANOVA. In some cases, the hypothesis tested suggested that a one-tailed repeated measures ANOVA would better

suit these data, and was utilized when appropriate. Non-normally distributed data were analyzed by rank repeated measures ANOVA (60). Zimmerman and Zumbo (60) determined that repeated measures ANOVA on ranks performs similarly to the Wilcoxon test and is good for small sample sizes. Student's t-tests and Tukey-Kramer tests were used as the post-hoc tests when a significant effect for diet and time effects were found. All statistics were determined with JMP 9.0.0 (SAS Institute Inc., Cary, NC) with $P < 0.05$ being considered significantly different.

Results

Feed intake

Feed intake was analyzed using average total feed consumption per week. As such, there were no significant difference in feed intake between the two diet groups over the acclimation period. However, feed intake increased with time over the acclimation period ($P < 0.0001$). Over the 7 week feeding trial, the FLX group consumed significantly more feed than the SUN group (81.3±10.2 and 74.8±9.3 g/bird/week, $P < 0.0001$). However, feed intake decreased over the 7 week period for both diet groups ($P < 0.0001$,Figure 3).

Body weight and condition score

No significant differences between the experimental groups for body weight or body condition score were observed up to day 70. No time effect was observed for body weight or body condition score (Table 3).

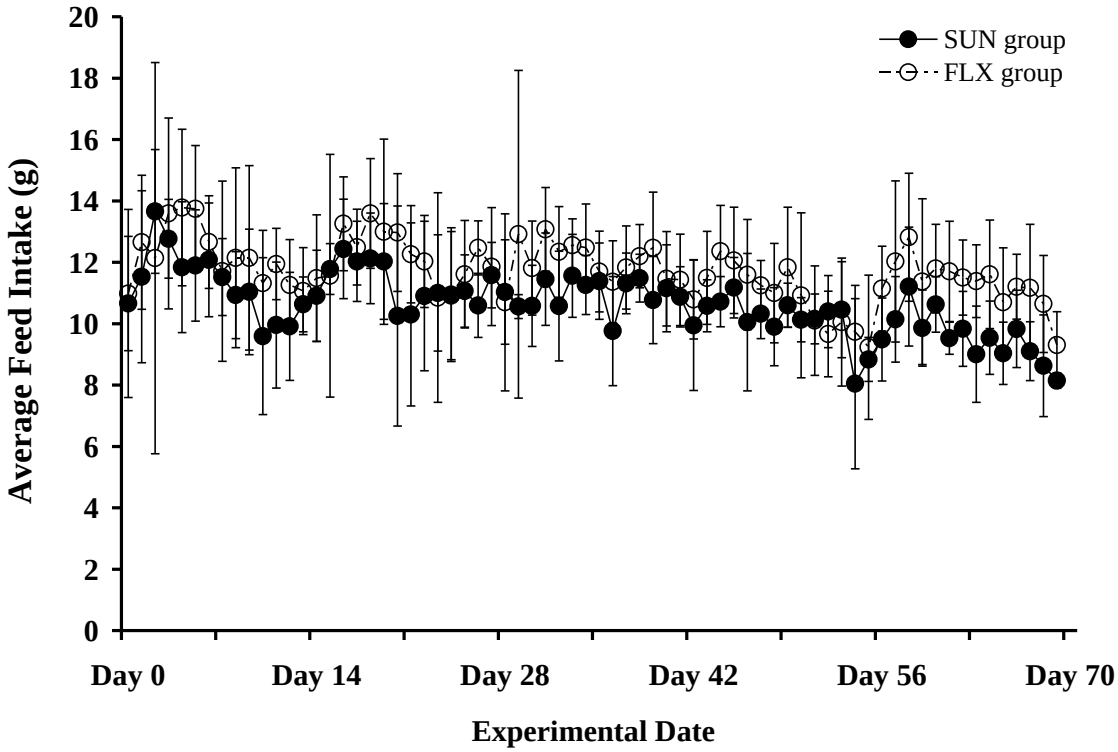

Figure 3. Feed intake over 70 days on the experimental diets.
SUN, sunflower seed diet; FLX, flaxseed diet.

Table 3. Body weight and body condition score (BCS) over the 70 day feeding trial.

	Week -5	Day 0	Day 7	Day 14	Day 28	Day 42	Day 56	Day 70
Body weight								
SUN	115.8 ± 15.4	112.8 ± 12.6	114.0 ± 12.0	115.0 ± 12.3	115.5 ± 11.4	116.0 ± 12.0	115.5 ± 13.8	115.2 ± 13.6
FLX	113.8 ± 11.9	109.6 ± 9.3	111.6 ± 10.8	112.8 ± 9.6	113.4 ± 9.5	114.2 ± 10.8	113.0 ± 10.9	115.3 ± 10.5
BCS								
SUN	3.3 ± 1.0	3.2 ± 0.7	3.2 ± 0.7	3.0 ± 0.6	3.1 ± 0.7	3.4 ± 0.8	3.2 ± 0.8	3.4 ± 0.5
FLX	3.6 ± 0.8	3.4 ± 0.7	3.5 ± 0.7	3.2 ± 0.6	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.5 ± 0.7

Values are expressed as mean ± SD with body weight expressed in g. There were no significant diet or time differences. SUN, sunflower seed diet; FLX, flaxseed diet; BCS, body condition score.

Plasma lipoproteins

A few of the birds were observed to have portomicrons in their plasma despite three hour removal of feed prior to sampling. However, no significant differences between diet groups were found for this fraction. The diet groups additionally had no significant differences among plasma beta, pre-beta, or alpha lipoprotein amounts when expressed either as a percentage of total lipoproteins or plasma cholesterol distributions. These data were therefore pooled resulting in mean plasma values for the lipoprotein classes for all birds expressed as percentage of total lipoproteins (mean ± SD) as follows: portomicrons 5.1% ± 2.7, pre- ß (VLDL) 26.6% ± 1.9, ß (LDL) 19.6% ± 2.1, and α (HDL) 54.0% ± 1.6. When expressed as a percentage, a time effect was only observed for ß ($P = 0.0007$). Percent ß decreased until day 28, then increased. A correlation between TC and % ß was not found. A time effect was observed for all lipoprotein fractions when expressed as LP-C due to cholesterol peaking at day 28 before returning to baseline.

Plasma lipoprotein density profile

The FLX group had significantly decreased relative percent HDL_{2b} ($P < 0.001$) compared to the SUN group at day 70 (Table 4). The FLX group also had significantly increased relative percent HDL_{3a} at day 28 ($P = 0.027$); however, this was no longer observed at day 70. When expressed as plasma cholesterol distributions, LDL_3 was higher in the FLX group on day 70 ($P = 0.05$). In the SUN group, relative percent HDL_{3a} was decreased at day 28 and then increased to its baseline value by day 70 ($P = 0.01$). This same lipoprotein alteration over time was observed for the absolute amounts of

HDL_{3a} and HDL_{3b} in the SUN group ($P = 0.03$ and $P = 0.05$, respectively). The relative percent HDL_{3c} significantly increased with time in the FLX group ($P = 0.01$). There was a time effect with the absolute amounts of LDL_4, LDL_5, and HDL_{2b} significantly decreasing in the FLX group over the course of the study.

It is also of interest that the HDL peak density value was shifted between the SUN group and the FLX group. By day 70, a statistically significant higher total HDL peak density value was found in the FLX group compared to the SUN group (1.097 g/ml FLX group vs. 1.095 g/ml SUN group, $P = 0.028$, Figure 4). However, both values are within the reported density range of HDL_{2a} (1.091-1.110 g/ml) for humans (Rifai et al 2000).

Total cholesterol, free cholesterol, esterified cholesterol, and triacylglycerols

No significant diet differences between the two experimental groups were observed for TC, FC, EC, or TAG. However, a time effect was observed for all of these measurements ($P < 0.0001$ for all). Total cholesterol, EC, and FC peaked at day 28 and then returned to baseline after slightly overshooting at day 56 (Figure 5). Triacylglycerols decreased over the acclimation period and then gradually increased over the course of the study, but never increased to baseline values ($P < 0.0001$).

Plasma phospholipid fatty acids

Plasma phospholipid fatty acid profiles were similar between both diet groups at day 0. At day 70, the FLX group had significantly higher plasma phospholipid fatty acids including myristic (14:0), γ-linolenic acid (18:3n-6), ALA (18:3n-3), EPA (20:5n-3), docosapentaenoic acid (22:5n-3, DPA), and DHA (22:6n-3). The SUN group

Table 4. Lipoprotein subfractions for birds fed the experimental diets for 70 days as determined by density gradient ultracentrifugation.

Lipoprotein Subclass	FLX	SUN
VLDL	2.0 ± 1.5	1.5 ± 1.0
LDL_1	0.6 ± 0.4	0.6 ± 0.3
LDL_2	1.4 ± 0.5	1.3 ± 0.6
LDL_3	2.7 ± 0.7	2.2 ± 1.0
LDL_4	4.3 ± 1.0	3.5 ± 0.9
LDL_5	10.4 ± 2.0	10.3 ± 1.7
Total LDL	19.5 ± 3.7	17.8 ± 3.9
HDL_{2b}	47.6 ± 1.5 *	51.6 ± 2.6
HDL_{2a}	21.7 ± 3.2	20.4 ± 3.2
HDL_{3a}	5.8 ± 1.3	5.1 ± 1.0
HDL_{3b}	2.0 ± 0.4	2.1 ± 0.5
HDL_{3c}	1.4 ± 0.3	1.6 ± 0.8
Total HDL	78.5 ± 4.9	80.6 ± 4.0

All values are expressed as percent of total lipoproteins (mean ± SD). Rows with an asterisk are significantly different between experimental groups ($P < 0.05$). FLX, flaxseed diet; SUN, sunflower seed diet; VLDL, very low-density lipoprotein; LDL, Low-density lipoprotein; HDL, high-density lipoprotein.

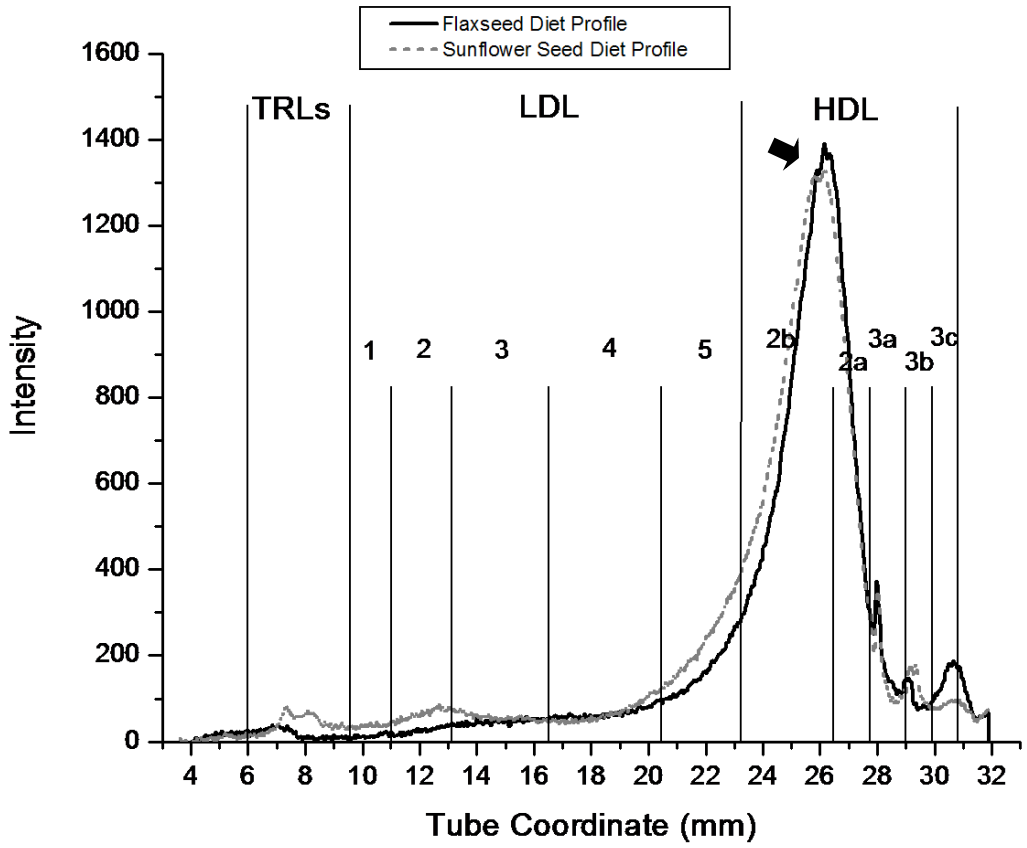

Figure 4. Lipoprotein distribution from density gradient ultracentrifugation of one representative bird from each experimental group showing the density peak shift of the total high-density lipoprotein (HDL) fraction at day 70 (3FOK08006 flaxseed group and JLL9TX345 sunflower seed group).

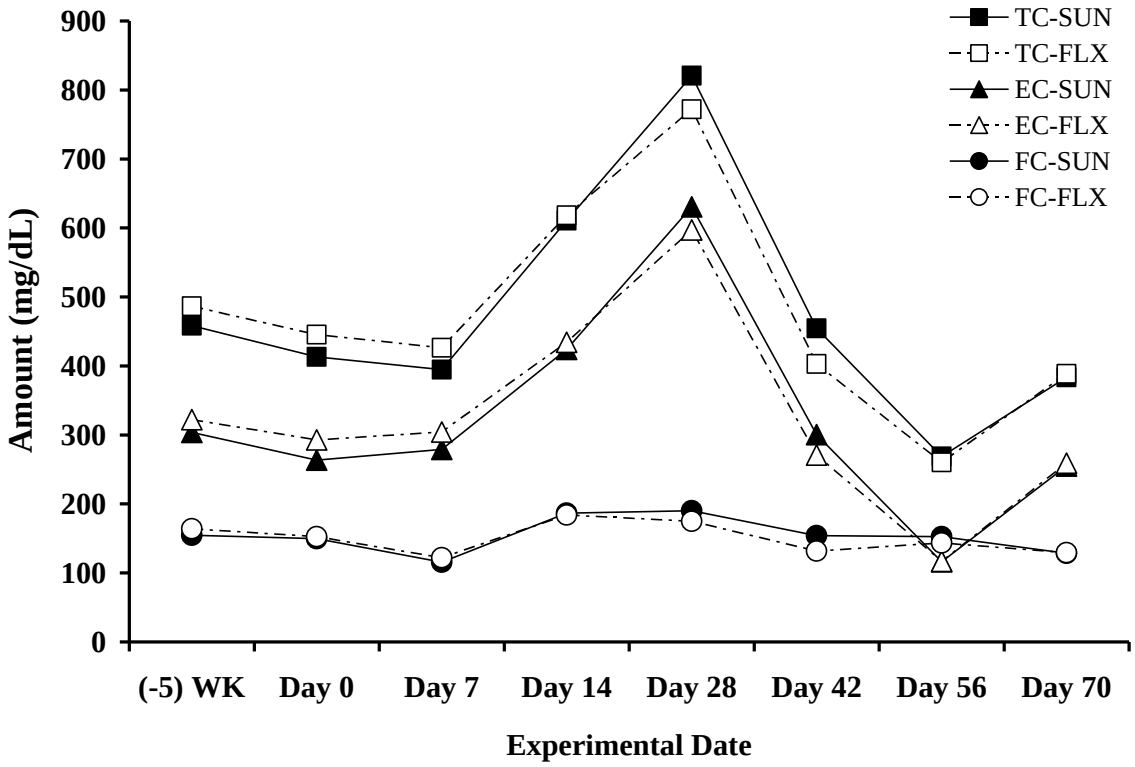

Figure 5. Total cholesterol (TC), esterified cholesterol (EC), and free cholesterol (FC) over the 70 day feeding trial. FLX, flaxseed diet; SUN, sunflower seed diet.

Table 5. Plasma phospholipid fatty acid profiles of birds fed the experimental diets for 70 days.[1]

	Fatty Acid	FLX	SUN
Saturated fatty acids			
	14:0	0.21 ± 0.1	0.18 ± 0.1
	15:0	0.06 ± 0.1	0.15 ± 0.1
	16:0	17.9 ± 2.5	20.1 ± 2.9
	17:0	0.32 ± 0.1	0.25 ± 0.1
	18:0	29.4 ± 3.1	27.7 ± 4.9
	20:0	0.43 ± 0.2	0.43 ± 0.1
	22:0	0.26 ± 0.1	0.29 ± 0.1
	24:0	0.33 ± 0.1	0.28 ± 0.1
	Total SFA	48.9 ± 4.0	49.3 ± 6.6
Monounsaturated fatty acids			
	16:1	0.53 ± 0.2	0.43 ± 0.2
	18:1n-9	14.4 ± 2.4	11.8 ±2.7
	18:1n-7	1.9 ± 0.9	1.7 ± 0.7
	20:1	0.21 ± 0.1	0.20 ± 0.1
	24:1	0.44 ± 0.2	0.48 ± 0.1
	Total MUFA	17.5 ± 2.5 *	14.6 ± 2.4
n-6 Polyunsaturated fatty acids			
	18:2n-6 (LA)	16.8 ± 3.2	18.2 ± 3.9
	18:3n-6	0.22 ± 0.0	0.16 ± 0.0
	20:2n-6	0.32 ± 0.1	0.41 ± 0.1
	20:3n-6Δ7	0.02 ± 0.0	0.03 ± 0.1
	20:3n-6Δ8	0.47 ± 0.1	0.57 ± 0.1
	20:4n-6 (AA)	4.5 ± 1.1 *	11.7 ± 2.7
	22:4n-6	1.2 ± 0.3	1.2 ± 0.2
	Total n-6 PUFA	23.6 ± 4.1 *	32.3 ± 6.4
n-3 Polyunsaturated fatty acids			
	18:3n-3 (ALA)	1.8 ± 0.6 *	0.17 ± 0.1
	20:5n-3 (EPA)	4.2 ± 0.9 *	0.03 ± 0.1
	22:5n-3	2.06 ± 0.8	0.3 ± 0.1
	22:6n-3 (DHA)	2.4 ± 0.6 *	1.8 ± 0.6
	Total n-3 PUFA	10.5 ± 1.6 *	2.3 ± 0.6
	Total PUFA	34.0 ± 5.0	34.5 ± 6.1

[1]All values are expressed as percent of total plasma phospholipid fatty acids (mean ± SD). Rows with an asterisk are significantly different between experimental groups ($P < 0.05$). FLX, flaxseed diet; SUN, sunflower seed diet; SFA, saturated fatty acids, MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

displayed significantly higher plasma phospholipid fatty acids 15:0, 20:2n-6, and arachidonic acid (20:4n-6, AA, Table 5).

Discussion

The time effect observed for feed intake which increased over the acclimation period may have been due to the birds accepting the new pelleted diets as the proportions of the two feeds changed. While parrots have been observed to be neophobic, especially with new feeds (49) this did not appear to be the case for the Monk parrots in this feeding trial. Lumeij and colleagues (47) saw an increase in feed intake after switching parrots from a seed mixture diet to an extruded diet. It may be possible that a similar increase occurred when the birds were switched from an extruded diet to a pelleted one. The Monk parrots may have found the pelleted diets utilized in this study more palatable than the extruded diet. The pelleted diets had a higher moisture content than the extruded diet and also contained molasses. It is possible that the decrease in feed intake over the 7 week feed trial after acclimation could have been due to an increase in ambient temperature which was observed towards the end of the study. Decreased or no significant effects on feed intake have been recorded in previous studies investigating the effects of dietary flaxseed in chickens (52, 54). However, an increase in feed intake for Monk parrots on the FLX diet occurred in this study. It is possible that the metabolizable energy content of the FLX diet was underestimated. Differences in dietary fiber composition of flaxseeds versus sunflower seeds could have also resulted in alterations in digestibility between the two diets. It is also possible that Monk parrots do

not have an aversion to and possibly a preference for the taste of flaxseed. Thus, Monk parrots and chickens may have different physiological responses to dietary flaxseed.

In this study, no significant differences in body weight were observed. A study by Ansenberger and colleagues (29) observed that the inclusion of 10% flaxseed in the diet improved the ability of commercial laying hens to maintain their body weights (29). However, several other studies in chickens noted reductions in body weight gain at dietary inclusion levels of flaxseed as low as 10% (53, 62). Because weight loss was not observed in this study, it is possible that other compounds (including mucilage, trypsin inhibitors, cyanogenic glucosides, and phytic acid) present in ground flaxseed that result in lower body weights in chickens may be ineffective in Monk parrots.

Similar to body weight, no significant time effect occurred for BCS. The BCS values straddle a range of weights, so small variations were likely minimized and thus prevented the observation of any significant differences. Additionally, this BCS method is characterized by palpation of muscle mass and subcutaneous fat in only one region, thus any changes in other fat depots were not accounted for by BCS.

The predominate lipoprotein cholesterol fraction in Monk parrots was α. This is similar to poultry and dogs, but unlike humans whose predominate lipoprotein cholesterol fraction is ß (63). All of lipoprotein-cholesterol fraction values as determined by electrophoresis followed the changes observed for plasma TC, which was expected as the LP-C values are determined using plasma TC values. However, when expressed as a percent of all lipoproteins, they all remained fairly constant, yet a significant decrease up to day 28 followed by an increase to baseline values did occur for ß. It is possible that

the ß lipoprotein fraction is inversely related with TC; however, the power in this study was not large enough to determine a correlation. McAlister and colleagues (64) commented that dogs have minimal accumulations of ß lipoproteins when fed high fat diets, which is one likely reason they are resistant to developing atherosclerosis (41). However, in the present study, Monk parrots, which are prone to developing atherosclerosis, had lowered relative percentage of ß lipoproteins when initially fed the higher fat diet. Maintaining low ß lipoprotein levels is important due to the oxidation of this lipoprotein fraction increasing the risk of developing atherosclerosis. However, low ß lipoprotein levels were observed in both dogs (resistant to atherosclerosis) and Monk parrots (prone to atherosclerosis) fed a high fat diet. Thus the ability to maintain ß lipoprotein levels when a high fat diet is fed to a predominately HDL animal alone may have a limited or no role in preventing the development of atherosclerosis. Although it is likely still important when considered in conjunction with other risk factors. In addition, dogs limited their production of pre-ß and removed cholesterol rich lipoproteins and remnants from the plasma when fed a high fat diet (41). The Monk parrots did not show increased relative percentage of the pre-ß fraction when fed the experimental diets containing higher dietary fat. It is important to note that a comparable increase in fat percentage of energy from the normal diet to the higher fat diet occurred in both the dog and Monk parrot studies. The dog diet increased from 22 en% fat to 57 en% fat (2.6 fold increase) (64) while the Monk parrot diet increased from 11.6 en% fat to 24.8 en% fat in SUN and 26.9 en% fat in FLX (2.1 and 2.3 fold increases, respectively). However, it is possible that a further increase in dietary fat may result in changes in the lipoprotein

fractions in Monk parrots and should be investigated. It is unknown if Monk parrots remove cholesterol rich lipoproteins and remnants similar to dogs (41) when fed a high fat diet, thus this also needs to be investigated further to see if this is a possible mechanism by which atherosclerosis can be prevented in other species.

Specific reasons for the increased total HDL peak density with the FLX diet are unknown at this time. One possibility is that it may be due to differences in molar mass of the long chain polyunsaturated fatty acids (PUFA) comprising the lipoprotein. For example, LA and ALA have similar molar masses (280.45 g/mol and 278.43 g/mol, respectively). Additionally, AA and EPA have similar molar masses (304.47 g/mol and 302.45 g/mol, respectively). However, DHA, which also accumulated in the FLX group, has a molar mass of 328.49 g/mol. An increase in the lipid mass from highly unsaturated long chain fatty acids comprising a lipoprotein particle, at similar particle size, may thus partially explain an increased particle density by a small, yet statistically significant amount as was seen in the FLX group. Alternatively, variation in peak density may have also been due to differences in the molecular shape of the long chain PUFA. Consequently, their three-dimensional structure and molecular orientation in the lipoprotein may be affected, especially given the “hair-pin like” three-dimensional configurations of the long chain PUFA fatty acids. Therefore, the density shift may be indicative of incorporation of long chain n-3 fatty acids, specifically DHA, into lipoprotein fractions. However, further physico-chemical evaluations of these possibilities are warranted. Consistent with the above possibilities are the findings of Salem and Niebylski (65) who have observed that the biophysical properties of various

polyenoic species of phospholipids are distinct and that the free volume of the acyl chain is maximal with DHA.

It should also be noted that the percentage HDL fraction as determined via electrophoresis was less than that obtained by density gradient ultracentrifugation. Lipoprotein electrophoresis is less quantitative overall than density gradient ultracentrifugation due, in part, to the differential staining ability of lipid fractions with the lipophilic stain used in the electrophoresis procedure. Electrophoresis also separates lipoproteins based on charge whereas density gradient ultracentrifugation characterizes the fractions by hydrated density. Thus some degree of caution should be used when directly comparing findings from these two techniques. Additionally, a study of goose plasma lipoproteins within the density range 1.034-1.055 g/ml were observed to have both α (HDL-C) and ß (LDL-C) mobility on electrophoresis (66). Thus, the possibility exists that some lipoproteins classified as HDL via electrophoresis in the present study are actually a type of LDL. However, because apo-protein composition of lipoprotein fractions was not performed, it remains to be determined whether this was the case. In any event, findings from both lipoprotein methods indicate that Monk parrots are similar to other known bird species with HDL as the predominate lipoprotein class.

The slightly higher LDL_3 subfraction (1.029-1.039 g/ml) in the FLX group at day 70 may have also contained part of an HDL fraction, especially given the observation that both LDL and HDL-like particles have been found within this density range in goose plasma (65). Such overlap of LDL and HDL does not occur in humans by these techniques, but comparative avian differences have not been studied in this regard to

date. Furthermore, the physiological implications of these findings in birds are also unknown. The shift in HDL peak density was initially noted at day 28, but became more evident at day 70. Thus, the possibility exists that further shifts may have continued had the diets been fed for a longer period.

It is of interest that the effect of feeding FLX in birds, which increased plasma ALA and its longer chain derivatives, had an opposite effect on lipoprotein subfraction distributions compared to humans (37). Male human subjects, aged 20-25 years, supplemented with 1.25 g/d tuna oil (25% eicosapentaenoic acid and 13% docosahexaenoic acid) for 2 weeks showed significantly decreased HDL_{3c} and HDL_{3b} and increased HDL_{2b}. In humans, this lipoprotein subfraction pattern is known as the longevity pattern and appears to be associated with a low risk of atherosclerosis (37). However, in the present study, when Monk parrots were fed an ALA enriched diet containing 10% ground flaxseed, the relative percent of HDL_{3c} increased while both the relative percent and absolute amount of HDL_{2b} decreased and HDL_{3b} remained constant. These findings suggest that other risk factors for atherosclerosis may exist in Monk Parrots compared to humans. Nonetheless, the potential for an anti-inflammatory effect due to the conversion of ALA to its longer chain n-3 derivatives may exist. If so, this dietary component may help mitigate atherosclerotic risk in this avian species.

The experimental diets contained a higher percent of fat than the ACC diet (Table 1). Thus the peak TC, FC, and EC values seen day 28 followed by their return to baseline may have resulted from adaptation of the birds to the higher dietary fat content of the experimental diets. The decrease in TAG over the acclimation period followed by

a gradual increase toward baseline could be due to changes in the percent fat of the diets, although the ACC diet had a similar fat content to Zupreem Natural® (Table 1). It is interesting to note that the ACC diet had a lower PUFA:SFA ratio than Zupreem Natural® and both experimental diets (2.6 ACC, 4.6 Zupreem Natural®, 4.1 FLX, and 3.9 SUN). It is unknown at this time if the PUFA:SFA ratio has a significant impact on plasma TAG levels.

It is important to note that the diets used in the present study did not contain any long chain n-3 or n-6 fatty acids. Thus, the plasma fatty acid results demonstrate that Monk parrots are capable of converting LA to the longer chain n-6 derivatives including AA along with converting ALA to the longer chain n-3 derivatives including both EPA and DHA. This ability especially contrasts with humans and most other mammals which do not efficiently convert ALA to DHA (21). Furthermore, accumulation of these long chain n-3 fatty acid derivatives were associated with a decrease in AA in the plasma phospholipid fraction. These results demonstrate that plasma phospholipid AA can be replaced by long chain n-3 fatty acid derivatives, especially EPA, and presumably similarly enriched avian tissues as observed in mammals. If so, then a less pro-inflammatory state may exist in birds fed ALA in the form of flaxseed compared to diets employing SUN. Decreasing the pro-inflammatory state in the body may help decrease the risk of developing chronic, progressive diseases having an inflammatory component such as atherosclerosis (24). This observation may be important because SUN are often used in feeds and as treats for caged birds whose longevity places them at risk for several chronic inflammatory diseases.

CHAPTER III

OXIDATIVE STATUS OF MONK PARROTS FED POLYUNSATURATED FATTY ACIDS

Introduction

Linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) compete for the same desaturase and elongase enzymes in order to produce their respective long chain derivatives (67). In addition, it has been shown in rats and humans that a "saturable" hyperbolic relationship exists between the incorporation of long chain n-6 and n-3 derivatives and the dietary inclusion levels of precursors LA and ALA (67, 68). It is likely that similar effects occur in birds. Thus the dose and possible saturation response of increasing dietary ALA at constant LA concentration on lipid metabolism was evaluated. Biomarkers of oxidation were also evaluated in this study. Eicosanoids (prostaglandins, prostacyclins, thromboxanes, and leukotrienes) are responsible for regulating the immune and inflammatory responses in the body. Eicosanoids derived from n-3 fatty acids are less potent than those from n-6 fatty acids (23-25). A major source of body inflammation during the development of atherosclerosis is due to oxidation, especially of low-density lipoproteins (LDL, 1). Thus a larger proportion of eicoisanoids derived from dietary n-3 fatty acids may decrease the inflammatory response to oxidized LDL in the body and potentially decrease the development of atherosclerosis (24). Furthermore, because oxidation plays a major role in the development of atherosclerosis, superoxide dismutase, catalase, and malondialdehyde in

erythrocyte lysates were assessed in this study as indicators of body oxidation. The effects of providing dietary n-3 as docosahexaenoic acid (22:6n-3, DHA) directly rather than ALA to Monk parrots on lipid metabolism and body oxidative status was also evaluated. In addition, the possibility of retro-conversion of DHA to eicosapentaenoic acid (20:5n-3, EPA) in the Monk parrot was also investigated.

Materials and Methods

The study utilized 29 adult Monk Parrots (*Myiopsitta monachus*) housed at the Schubot Exotic Bird Health Center at Texas A&M University. Each bird received a physical examination, including body weight and condition score assessment, gender determination, and was permanently identified with a metal leg band prior to starting the study. All birds were maintained in 0.61 m x 1.22 m x 1.83 m (2' x 4' x 6') cages in pairs (except for two birds, due to the original odd number of birds available for the study and the subsequent death of a cage-mate during the acclimation period). The birds were housed in a covered facility with two steel mesh walls, which provided protection from inclement weather. In the winter, heavy curtains were secured over the steel mesh walls and heat lamps were utilized when the temperature fell below 4.4°C (40°F). When the temperature rose above 32.2°C (90°F), a swamp cooler and large wall fans were used to cool the aviary. Birds were provided feed and water *ad libitum*.

Birds were transitioned onto a pelleted, complete, and balanced acclimation diet (ACC, corn-soybean-rice based) for 5 to 6 weeks prior to feeding the study diets (-5/-6 weeks). Diet ingredients consisted of corn, soybean meal, alfalfa, broken rice, molasses, vitamin pre-mix, and mineral pre-mix (Table 6). The total

acclimation period lasted from February 28, 2011 through April 4-11, 2011, due to staggered start dates. For the first six days of the acclimation period, the birds were fed 75% Zupreem Natural® and 25% ACC diet. The following six days consisted of the birds being fed 50% Zupreem Natural® and 50% ACC diet. The next 6 days was comprised of 25% Zupreem Natural® and 75% ACC diet. For the remainder of the acclimation period, birds were fed 100% ACC diet. At the beginning of the dietary test period (day 0) the birds were divided into four groups [Low ALA (LALA), Medium ALA (MALA), High ALA (HALA), or DHA] and fed their respective experimental diet, again as a complete and balanced pelleted feed (Tables 6 and 7). The LALA diet also served as the acclimation diet. The ALA experimental diets (Low, Medium, and High) were prepared by substituting linseed oil for sunflower oil in order to adjust the ALA levels in the diet. The DHA experimental diet included DHA Gold (Martek Biosciences Corporation, Columbia, MD), an algae product, with minor modifications to ingredient amounts of the ALA diets as necessary to be complete, balanced, and isocaloric with the other experimental diets (Table 6). The amount of feed offered (20 g/bird/day) was determined to be sufficient from the preliminary study. Birds in the MALA and HALA groups were fed their respective diets for 28 days. The Monk parrots in the LALA and DHA groups continued to be fed their respective diets until day 63 as part of a separate longer study to be described in Chapter 4.

Due to the death of one bird assigned to the DHA group from causes unrelated to the study on day -2, 8 birds in the HALA group, 8 birds in the MALA group, 7 birds in the LALA group, and 5 birds in the DHA group completed the feeding trial.

Table 6. Diet ingredients and nutrient composition of the low α-linolenic acid (LALA), medium α-linolenic acid (MALA), high α-linolenic acid (HALA), and docosahexaenoic acid (DHA) diets.

Nutrient	LALA	MALA	HALA	DHA
Corn, Yellow	45.3	45.3	45.3	47.0
Soybean Meal, Dehulled (48%)	18.2	18.2	18.2	16.2
Rice, Broken	17.3	17.3	17.3	15.5
Molasses, Dehydrated	2.0	2.0	2.0	2.0
Alfalfa, Meal (17%)	9.0	9.0	9.0	9.0
High-Oleic Sunflower Oil	4.0	2.4	0.0	0.9
Linseed Oil	0.0	1.6	4.0	0.0
Martek DHA Gold®	0.0	0.0	0.0	5.5
DL-Methionine (98%)	0.2	0.2	0.2	0.2
L-Lysine Hydrochloride	0.01	0.01	0.01	0.06
Limestone	1.3	1.3	1.3	1.3
Mono- + Dicalcium Phosphate	1.9	1.9	1.9	1.9
Sodium Chloride	0.15	0.15	0.15	0.13
Mineral Premix	0.05	0.05	0.05	0.05
Vitamin Premix	0.25	0.25	0.25	0.25
Moisture	10.4	10.8	10.5	12.2
Crude Protein	14.7	14.9	14.7	16.1
Crude Fiber	5.4	6.1	5.1	5.2
Ash	5.3	5.5	5.4	5.4
Fat	6.5	7.2	6.8	6.3

The LALA diet also served as the acclimation diet. Nutrient composition values are on an “as fed” basis. The vitamin and mineral premixes are described in Table 1 (Chapter II).

Table 7. Fatty acid composition of the low α-linolenic acid (LALA), medium α-linolenic acid (MALA), high α-linolenic acid (HALA), and docosahexaenoic acid (DHA) diets.

	Fatty Acid	LALA	MALA	HALA	DHA
Saturated fatty acids					
	12:0	0.0	0.1	0.0	0.0
	14:0	0.0	0.1	0.0	0.5
	16:0	7.4	7.5	9.0	7.3
	18:0	3.6	4.0	4.3	0.7
	20:0	0.5	0.5	0.4	0.4
	22:0	0.8	0.7	0.5	0.4
	24:0	0.4	0.4	0.3	0.0
	Total SFA	12.6	13.1	14.6	9.2
Monounsaturated fatty acids					
	16:1	0.2	0.2	0.1	0.3
	18:1n-9	58.2	44.8	33.5	15.3
	18:1n-7	0.9	0.7	1.3	8.8
	20:1	0.3	0.3	0.2	0.5
	Total MUFA	59.5	46.1	35.2	25.0
n-6 Polyunsaturated fatty acids					
	18:2n-6	26.6	28.1	29.7	25.5
	20:3n-6	0.0	0.0	0.0	0.1
	20:4n-6	0.0	0.0	0.0	1.1
	22:4n-6	0.0	0.0	0.0	10.3
	Total n-6 PUFA	26.6	28.1	29.7	37.0
n-3 Polyunsaturated fatty acids					
	18:3n-3	1.3	12.3	19.9	0.9
	22:5n-3	0.0	0.0	0.0	0.7
	22:6n-3	0.0	0.0	0.0	25.2
	Total n-3 PUFA	1.3	12.3	19.9	26.8
	Total PUFA	27.9	40.4	49.6	63.9
	PUFA:SFA Ratio	2.2	3.1	3.4	6.9

Values are expressed as a percentage of total fatty acids. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Feed intake

Feed intake was assessed following the protocol described in Chapter II. Feed intake was collected 5 days per week beginning with day 0 for this study.

Body weight and body condition scores

Body weights were recorded once a week at the same time of day beginning at -5/-6 weeks following the method described in Chapter II.

Body condition scores were analyzed on blood sample collection days (days 0, 28, and 63). Body condition was determined also as previously described in Chapter II.

Blood collection

Blood samples were obtained following the protocol as previously mentioned (Chapter II) on days 0 and 28. An additional blood sample at day 63 was obtained from birds in the LALA and DHA groups. Following slow speed centrifugation, the plasma was placed in microtainers for storage in a -80°C freezer and erythrocytes were prepared as described below.

Erythrocyte preparations

The erythrocytes were washed prior to storage at -80°C. Five hundred μL of 0.9% NaCl was added to the microtainer containing the sedimented erythrocytes. The microtainer was gently inverted until the erythrocytes were in solution after the cap had been secured. The solution was then transferred to a 12 mL teflon-lined screw-capped test tube. This was repeated 2 more times to ensure all of the erythrocytes were transferred to the test tube. An additional 5 mL of 0.9% NaCl was added to the test tube which was then gently inverted. It was next centrifuged at 2,622 rpm (1,600 x g) at 4°C for 10 minutes. The supernatant was discarded following centrifugation. The process

was repeated three more times with 6 mL of 0.9% NaCl added to the test tube each time. A total of 500 µL (2 additions of 250 uL) 0.9% NaCl was used to transfer the erythrocytes from the 12 mL test tube to a clean microtainer. It was then centrifuged at 2,622 rpm (1,600 x g) at 4°C for 10 minutes. The supernatant was removed and 20 µL 0.9% NaCl was added to the microtainer. Nitrogen was streamed over the surface and the samples were stored in a -80°C freezer.

Preparing erythrocyte lysate

The washed erythrocytes were thawed and 500 µL ice-cold HPLC grade water was added to the microtainer. The samples were mixed using a vortex mixer and transferred to 12 mL teflon-lined screw-capped test tubes. Then, 1.5 mL ice-cold HPLC grade water was added to each test tube. Tube contents were then mixed and centrifuged at 4,700 rpm (5,142 x g) at 4°C for 30 minutes. Following centrifugation, the supernatant was aliquoted into clean microtainers. Nitrogen gas was streamed over the surface of each sample. Erythrocytes were lysed immediately prior to performing the malondialdehyde assay. Erythrocyte lysates were stored at -80°C prior to performing the BCA protein, catalase, and superoxide dismutase assays, essentially according to Iraz et al (69).

Plasma phospholipid fatty acid profiles

Total lipids from Monk parrot plasma samples and diets were extracted following a modified protocol of Folch and colleagues (55, 56) as previously described (Chapter II).

Total plasma cholesterol, free cholesterol, esterified cholesterol, and triacylglycerol concentrations

Total cholesterol (TC), free cholesterol (FC), and triacylglycerol (TAG) concentrations were determined by colorimetric endpoint enzymatic assays according to Wright-Rodgers and colleagues (57) as previously described. Esterified cholesterol (EC) concentration was determined by subtracting FC concentration from TC concentration.

Malondialdehyde

Malondialdehyde (MDA) was measured in the erythrocyte lysates on days 0, 28, and 63 following a modified method described by Iraz and colleagues (69) with clarification from Esterbauer and Cheeseman (70). The erythrocyte lysate (200 µL) was reacted with an equal volume of 10% (w/v) trichloroacetic acid to precipitate the proteins. It was then mixed and centrifuged at 2,622 rpm (1,600 x g) at 4°C for 15 minutes. Following centrifugation, 150 µL of the supernatant or standard (0, 0.625, 1.25, 2.5, 5, and 10 µM) was placed in a clean microtainer. An equal volume of 0.67% (w/v) thiobarbituric acid was added to the microtainer. Tubes were then placed in a boiling water bath for 10 minutes. After allowing the samples to come to room temperature, they were pipetted into wells of a microtiter plate and the absorbance was measured (Molecular Devices Corporation, Sunnyvale, CA) at 550 nm and its accompanying software (SoftMax Pro, Molecular Devices Corporation, Sunnyvale, CA).

Catalase

Catalase activities (CAT) were determined at days 0, 28, and 63 using a Catalase Assay Kit (Cayman Chemical Company, Ann Arbor, MI). Samples were thawed and

diluted 1:500 (v/v) using sample buffer. Twenty µL sample, standard (0, 5, 15, 30, 45, 60, 75 µM formaldehyde), or diluted bovine liver catalase was added to each well. Then, 100 µL assay buffer, followed by 30 µL methanol, was added to each well. Next, 20 µL hydrogen peroxide was added to each well. The plate was covered and incubated at room temperature on a plate shaker (Junior Orbit Shaker; Lab-Line Instruments, Melrose Park, IL) for 20 minutes. Following incubation, 30 µL potassium hydroride followed by 30 µL purpald (chromagen) was added to each well. The plate was again covered and incubated at room temperature on a plate shaker for 10 minutes. Then 10 µL potassium periodate was added to each well. The plate was covered and incubated at room temperature on a plate shaker for 5 minutes. The absorbance was measured using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 550 nm and its accompanying software (SoftMax Pro, Molecular Devices Corporation, Sunnyvale, CA). See Appendix B for the equations used to determine formaldehyde concentration and catalase activity.

Superoxide dismutase

Superoxide dismutase activities (SOD) were determined at days 0, 28, and 63 using an assay kit by Cayman Chemical Company (Ann Arbor, MI). Erythrocyte lysate samples were thawed. and 0.2 µL sample and 9.8 µL sample buffer was pipetted into each well, resulting in 1:49 (v/v) diluted samples. Ten µL standard (0, 0.025, 0.05, 0.1, 0.15, 0.2, and 0.25 U/mL) was pipetted into standard wells. Two hundred µL radical detector followed by 20 µL xanthine oxidase was added to each well. The plate was covered and incubated at room temperature on a plate shaker (Junior Orbit Shaker; Lab-Line Instruments, Melrose Park, IL) for 20 minutes. The absorbance was measured using

a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at 450 nm and its accompanying software (SoftMax Pro, Molecular Devices Corporation, Sunnyvale, CA). The equation for determining superoxide dismutase activity is provided in Appendix C.

Erythrocyte protein concentration

Hemoglobin concentration is commonly used to normalize measured malondialdehyde values. However, avian erythrocytes are nucleated, thus it may be more accurate to normalize malondialdehyde values using total protein concentration of the erythrocyte lysates. For this reason, total protein concentrations of the erythrocyte lysates were measured using the Pierce® bicinchoninic acid (BCA) method. Protein values were additionally used to normalize measured catalase and superoxide dismutase activities. Normalized MDA values were expressed as μmoles MDA/g protein. Normalized CAT values were expressed as nmol CAT/min/mg protein. Normalized SOD values were expressed as U SOD/mg protein.

After thawing the samples, 25 μL standard or 1:49 (v/v) diluted sample was pipetted into each well. Standards (0, 25, 125, 250, 500, 750, 1000, 1500, 2000 μg/mL) were made from bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Then, 200 μL working stock solution was added to each well. The working stock was made by adding 50 μL Pierce® BCA Protein Assay Reagent A to 1 μL Pierce® BCA Protein Assay Reagent B (Thermo Scientific, Rockford, IL). Plates were shaken for 30 seconds, covered, and incubated at 37ºC for 30 minutes. Following incubation, samples were allowed to come to room temperature. The absorbance of each well was read at 550 nm by a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) and its

accompanying software (SoftMax® Pro, Molecular Devices Corporation, Sunnyvale, CA).

Fat and fatty acid composition of experimental diets

The fat content and fatty acid composition of the experimental diets were determined as described previously (Chapter II).

Statistical analyses

Data were analyzed for normality using the Shapiro-Wilks test. Normally distributed data were then analyzed by repeated measures ANOVA. In some cases, the hypothesis tested suggested a one-tailed repeated measures ANOVA would better suit the data, and was utilized when appropriate. Non-normally distributed data were analyzed by rank repeated measures ANOVA (60). Zimmerman and Zumbo (60) determined that repeated measures ANOVA on ranks performs similarly to the Wilcoxon test and is good for small sample sizes. One way ANOVA and Tukey-Kramer tests were used as the post-hoc test when a significant effect for diet and time effects were found through day 28. Because only two experimental groups continued to day 63, Student's t-test was used as the post-hoc test for significant diet and time effects when evaluating data obtained on day 63. All statistics were determined with JMP 9.0.0 (SAS Institute Inc., Cary, NC) with $P < 0.05$ being considered significantly different.

Results

Feed intake

Feed intake was analyzed using total feed consumed per week (5 days collected out of 7). Feed intake was significantly increased in the DHA group compared to the

three ALA groups through the first 28 days (58.7 ± 5.6 g feed/bird/5days DHA group, 53.7 ± 6.9 g feed/bird/5days LALA group, 52.4 ± 3.5 g feed/bird/5days MALA group, and 51.0 ± 3.9 g feed/bird/5days HALA group, $P < 0.0001$). There was no difference in feed intake between the three ALA groups. The significant increase in feed intake for the DHA group remained at the end of the study on day 63 ($P = 0.0007$). There was also a time effect for feed intake over the first 28 days as well as through day 63 ($P = 0.01$ and $P < 0.0001$, respectively, Figure 6). Feed intake steadily declined from week 6 through week 9.

Body weights and condition score

There was a time effect for body weight for data analyzed through both days 28 and 63. However, no significant differences between experimental dates were seen with post-hoc tests. The birds gained weight from week -6 until week -3, then lost weight until day 0. There were no diet effects on body weight. (Table 8) No significant effects were observed for BCS (Table 8).

Plasma phospholipid fatty acid profile

Plasma phospholipid fatty acid profiles were similar between all treatment groups at day 0 with the exception of the birds in the DHA group having higher amounts of 16:1 than the birds in the MALA group ($P < 0.05$).

At day 28, the MALA and HALA groups had significantly higher plasma phospholipid 18:0 combined with significantly lower 18:1n-9 than the LALA group ($P = 0.0002$ and $P = 0.0005$, respectively, Table 9). There were no differences between the three ALA groups with respect LA. However, significantly elevated ALA occurred in

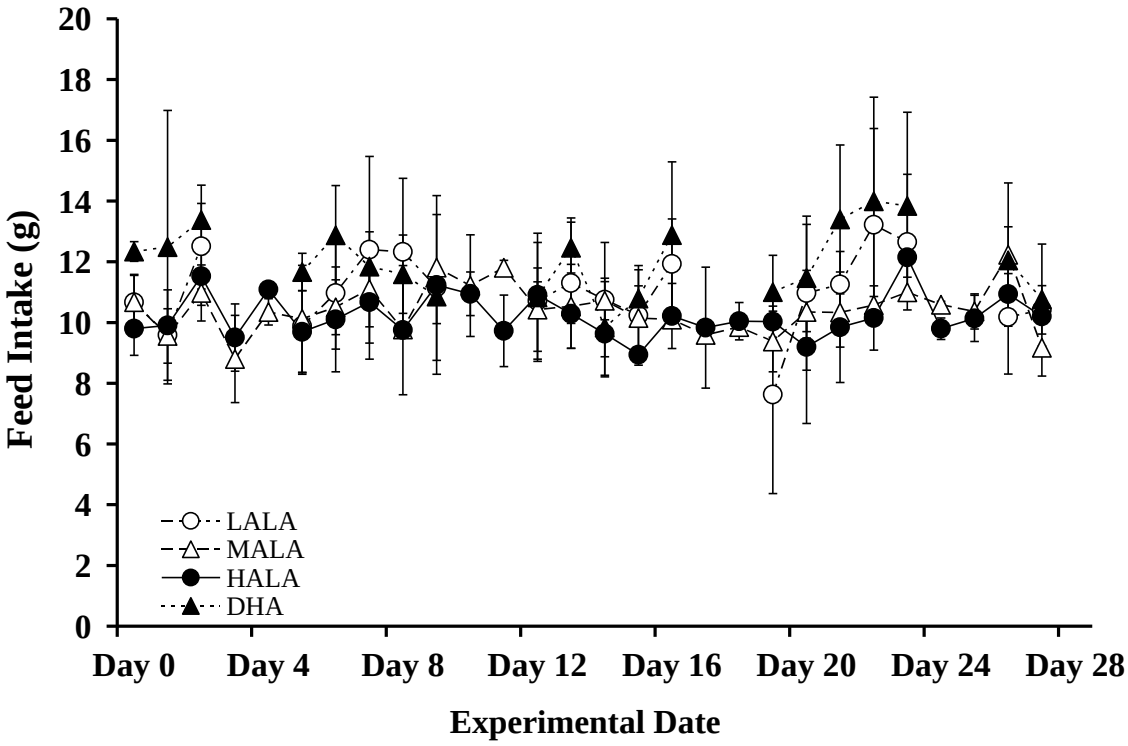

Figure 6. Feed intake over 28 days on the experimental diets.
LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet.

Table 8. Body weight and body condition score (BCS) over the 63 day feeding trial.[1]

	LALA	MALA	HALA	DHA
Body Weight (g)				
Week -6	108.8 ± 5.0	nd[2]	nd	115.5 ± 4.9
Week -5	110.3 ± 12.3	118.0 ± 11.7	112.0 ± 9.5	112.0 ± 7.7
Week -4	111.7 ± 12.4	121.8 ± 11.6	114.4 ± 8.9	114.3 ± 8.2
Week -3	114.0 ± 10.9	124.0 ±10.5	118.1 ± 9.9	113.7 ± 4.0
Week -2	110.9 ± 10.0	121.5 ± 9.2	113.6 ± 9.7	109.2 ± 3.3
Week -1	112.2 ± 8.6	118.0 ± 9.8	110.9 ± 9.3	113.0 ± 4.9
Day 0	113.8 ± 7.7	117.9 ± 9.2	113.8 ± 8.7	113.2 ± 3.1
Day 7	114.6 ± 7.3	117.0 ± 7.3	114.5 ± 9.7	115.0 ± 3.2
Day 14	114.3 ± 9.2	118.8 ± 9.9	116.0 ± 8.6	114.0 ± 1.4
Day 21	114.6 ± 10.1	120.3 ± 11.0	115.1 ± 10.9	113.6 ± 3.6
Day 28	116.6 ± 9.8	120.4 ± 11.3	115.4 ± 9.5	116.2 ± 3.1
Day 35	112.2 ± 5.2	nd	nd	117.2 ± 3.6
Day 42	115.5 ± 3.4	nd	nd	116.4 ± 4.3
Day 49	116.0 ± 2.1	nd	nd	117.6 ± 4.2
Day 56	113.3 ± 5.1	nd	nd	118.2 ± 4.9
Day 63	109.3 ± 3.4	nd	nd	116.2 ± 3.2
BCS				
Day 0	3.0 ± 0.6	2.9 ± 0.4	2.9 ± 0.6	3.0 ± 0.7
Day 28	3.3 ± 1.0	2.9 ± 0.8	3.4 ± 0.7	3.2 ± 0.4
Day 63	3.0 ± 0.6	nd	nd	3.6 ± 0.5

[1]Values are expressed as mean ± SD with body weight expressed in g. There were no significant diet or time effects. LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet
[2]nd, not determined.

Table 9. Plasma phospholipid fatty acid profiles of birds fed the experimental diets for 28 days.

	Fatty Acid	LALA	MALA	HALA	DHA
Saturated fatty acids					
	14:0	0.14 ± 0.1	0.18 ± 0.1	0.15 ± 0.1	0.20 ± 0.0
	15:0	0.07 ± 0.1	0.04 ± 0.1	0.23 ± 0.3	0.15 ± 0.1
	16:0	21.6 ± 2.7^{b}	21.2 ± 3.7^{b}	20.3 ± 2.5^{b}	27.3 ± 2.8^{a}
	17:0	0.17 ± 0.0	0.30 ± 0.1	0.32 ± 0.3	0.19 ± 0.0
	18:0	20.8 ± 2.3^{b}	29.6 ± 5.8^{a}	25.1 ± 4.6^{a}	20.2 ± 1.7^{b}
	20:0	0.29 ± 0.1	0.48 ± 0.2	0.33 ± 0.1	0.32 ± 0.1
	22:0	0.17 ± 0.1	0.32 ± 0.1	0.22 ± 0.1	0.27 ± 0.2
	24:0	0.32 ± 0.1^{b}	0.68 ± 0.9^{b}	0.37 ± 0.1^{b}	0.98 ± 0.3^{a}
	Total SFA	43.6 ± 2.9^{b}	52.8 ± 6.9^{a}	47.0 ± 4.6^{a,b}	49.6 ± 4.0^{a}
Monounsaturated fatty acids					
	16:1	1.0 ± 0.5	0.63 ± 0.4	0.78 ± 0.4	1.0 ± 0.4
	18:1n-9	20.7 ± 2.0^{a}	12.6 ± 5.7^{b}	15.5 ± 4.8^{b}	13.7 ± 2.1^{b}
	18:1n-7	2.3 ± 1.0	1.8 ± 1.2	2.0 ± 2.4	2.9 ± 2.6
	20:1	0.18 ± 0.1	0.18 ± 0.2	0.19 ± 0.1	0.10 ± 0.1
	24:1	0.82 ± 0.1	0.68 ± 0.3	0.93 ± 0.8	0.71 ± 0.1
	Total MUFA	25.0 ± 3.1^{a,b}	15.8 ± 6.6^{b}	19.4 ± 5.0^{a,b}	18.4 ± 3.1^{a}
n-6 Polyunsaturated fatty acids					
	18:2n-6	14.0 ± 1.6^{a}	14.9 ± 2.7^{a}	16.8 ± 2.4^{a}	6.9 ± 1.5^{b}
	18:3n-6	0.07 ± 0.1	0.13 ± 0.1	0.11 ± 0.1	0.00 ± 0.0
	20:2n-6	0.66 ± 0.1^{a}	0.34 ± 0.2^{b}	0.32 ± 0.1^{b}	0.11 ± 0.1^{c}
	20:3n-6Δ7	0.07 ± 0.1	0.09 ± 0.1	0.03 ± 0.0	0.00 ± 0.0
	20:3n-6Δ8	0.82 ± 0.1^{a}	0.73 ± 0.2^{a}	0.63 ± 0.1^{a}	0.31 ± 0.0^{b}
	20:4n-6	11.5 ± 2.4^{a}	7.9 ± 1.8^{b}	6.8 ± 1.1^{b}	7.2 ± 1.1^{b}
	22:4n-6	1.3 ± 0.2^{a}	0.57 ± 0.3^{b}	0.52 ± 0.2^{b}	1.8 ± 1.9^{a}
	Total n-6 PUFA	28.5 ± 3.7^{a}	24.7 ± 4.6^{a}	25.2 ± 3.3^{a}	16.3 ± 2.9^{b}
n-3 Polyunsaturated fatty acids					
	18:3n-3	0.11 ± 0.1^{b}	0.64 ± 0.3^{a}	0.92 ± 0.3^{a}	0.05 ± 0.1^{b}
	20:5n-3	0.20 ± 0.1^{b}	2.3 ± 0.8^{a}	3.2 ± 0.8^{a}	2.0 ± 0.6^{a}
	22:5n-3	0.26 ± 0.1^{b}	1.5 ± 1.2^{a}	1.4 ± 0.8^{a}	2.9 ± 2.3^{a}
	22:6n-3	1.8 ± 0.4^{b}	1.9 ± 0.9^{b}	2.3 ± 0.8^{b}	10.0 ± 2.1^{a}
	Total n-3 PUFA	2.4 ± 0.5^{c}	6.3 ± 1.3^{b}	7.8 ± 1.1^{b}	14.9 ± 4.2^{a}
	Total PUFA	30.9 ± 4.1	30.9 ± 5.6	33.0 ± 3.7	31.2 ± 5.0

All values are expressed as percent of total plasma phospholipid fatty acids (mean ± SD). Rows with different letters are significantly different between experimental diet groups ($P <$ 0.05). LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

the MALA and HALA groups compared to the LALA group ($P = 0.0001$).While not significantly different, the ALA value manifested a dose response between the MALA (0.64 rel%) and HALA (0.92 rel%) groups. The MALA and HALA groups also had significantly lower 20:2n-6 than the LALA group ($P < 0.0001$). However, further comparisons with the DHA diet suggested some 20:2n-6 accumulation had occurred in the MALA and HALA groups as the result of sparing the usage of LA to produce arachidonic acid (20:4n-6, AA). The LALA group had significantly higher AA and 22:4n-6 than the MALA or HALA groups ($P < 0.0001$ and $P = 0.001$, respectively). Significant accumulations of EPA and docosapentaenoic acid (22:5n-3, DPA) occurred in the MALA and HALA groups compared to the LALA group ($P < 0.0001$ for both). There were no significant differences in DHA between the three ALA diet groups. Furthermore, the DHA values suggest a dose response between the MALA (1.87 rel%) and HALA (2.33 rel%) groups.

When the plasma phospholipids of the DHA group were compared to the three ALA groups on day 28, more fatty acid differences were observed. The DHA group had significantly lower 16:0 than the three ALA groups ($P < 0.02$). The DHA group also had the same decrease in 18:1n-9 as observed in the MALA and HALA groups compared to the LALA group ($P = 0.0005$). However, the DHA group, unlike the MALA and HALA groups, had the same 18:0 as the LALA group ($P = 0.0002$). Significantly lower LA existed in the DHA group compared to the three ALA groups ($P < 0.0001$). The ALA in the DHA group was the same as the LALA, and less than the MALA and HALA groups ($P = 0.0001$). The DHA group also had less 20:2n-6 and 20:3n-6Δ8 than the three ALA

groups ($P < 0.0001$ and $P = 0.0003$, respectively). Arachidonic acid was also decreased from the LALA group and was similar to the MALA and HALA groups ($P < 0.0001$). However, 22:4n-6 was increased in the DHA group compared to the MALA and HALA groups to the same level as the LALA group ($P = 0.001$). Both EPA and DPA were significantly higher in the DHA group than the LALA group and were the same as the MALA and HALA groups ($P < 0.0001$ for both). The DHA group also had significant accumulation of DHA in the plasma phospholipids and was greater than the three ALA groups ($P < 0.01$). A significant increase in the DHA group over the three ALA groups also occurred with 24:0 ($P = 0.002$).

On day 63, the DHA group had significantly higher plasma phospholipid fatty acids EPA, 22:0, DPA, DHA, and 24:0 (Table 10). The LALA group had significantly higher plasma phospholipid fatty acids 18:3n6, 20:1, 20:2n-6, 20:3n-6Δ8, 11, 14 (DGLA), and AA on day 63.

Total cholesterol, free cholesterol, esterified cholesterol, and triacylglycerols

A time effect was observed for TC, FC, EC, and TAG through both day 28 and day 63. A decrease in TC ($P = 0.0002$ and $P = 0.02$, respectively) and EC ($P < 0.0001$ and $P = 0.0005$, respectively) occurred through both day 28 and 63. Free cholesterol and TAG increased over the study period ($P = 0.02$ and $P = 0.0005$, respectively for day 28 and $P = 0.003$ and $P = 0.003$, respectively for day 63). However, there were no diet effects for any of these analytes (Table 11).

Malondialdehyde, catalase, and superoxide dismutase

There was a significant time effect for MDA due to increased values between days 28 and 63 ($P = 0.001$). However, no significant diet effects were observed (Table 12).

Catalase showed a significant increase on day 0 for the HALA group compared to the DHA group, but not the LALA or MALA groups ($P = 0.0259$, Table 12). However, no significant diet or time effects were observed at days 28 nor 63.

A time effect was seen for SOD through both days 28 ($P < 0.0001$) and 63 ($p = 0.0004$). Superoxide dismutase decreased between days 0 and 28. No further decreases occurred between days 28 and 63. There were no diet effects observed at any time point (Table 12).

Because the LALA diet served as the ACC diet, the birds in the LALA group actually consumed their diet approximately 28 days longer than the other birds (18 days transitioning from Zupreem Natural® to ACC diet and 25 days consuming 100% ACC diet). Thus at day 0, the LALA birds had actually already consumed their diet for approximately 28 days. Consequently, MDA, CAT, and SOD were compared using the actual number of days the diet had been consumed by each group using ANOVA for the time points representing days 0 (day 0') and 28 (day 28') and Students t-test for the equivalent of day 63 (day 63'). When doing so, the significant difference for CAT at day 0 remained for day 0' ($P<0.01$). At day 28', the LALA group had significantly higher SOD than the HALA or DHA groups, but not the MALA group. The DHA group had significantly higher MDA at day 63' than the LALA group.

Table 10. Plasma phospholipid fatty acid profiles of birds fed the experimental diets for 63 days.

	Fatty Acid	LALA	DHA
Saturated fatty acids			
	14:0	0.19 ± 0.1	0.14 ± 0.1
	15:0	0.14 ± 0.2	0.13 ± 0.2
	16:0	22.2 ± 3.2	22.5 ± 3.4
	17:0	0.32 ± 0.2	0.31 ± 0.2
	18:0	22.6 ± 4.0	25.2 ± 4.7
	20:0	0.43 ± 0.3	0.51 ± 0.3
	22:0	0.20 ± 0.2[b]	0.43 ± 0.2[a]
	24:0	0.67 ± 0.7[b]	0.94 ± 0.4[a]
	Total SFA	46.7 ± 4.8[b]	50.2 ± 3.5[a]
Monounsaturated fatty acids			
	16:1	0.79 ± 0.2	0.53 ± 0.4
	18:1n-9	17.3 ± 5.3	13.4 ± 4.1
	18:1n-7	3.0 ± 1.6	1.7 ± 0.4
	20:1	0.28 ± 0.1[a]	0.16 ± 0.0[b]
	24:1	0.86 ± 0.2	0.77 ± 0.2
	Total MUFA	22.1 ± 4.2[a]	16.6 ± 4.4[b]
n-6 Polyunsaturated fatty acids			
	18:2n-6	13.5 ± 2.3[a]	8.2 ± 1.3[b]
	18:3n-6	0.05 ± 0.1	0.03 ± 0.1
	20:2n-6	0.64 ± 0.1[a]	0.22 ± 0.1[b]
	20:3n-6Δ7	0.04 ± 0.1	0.00 ± 0.0
	20:3n-6Δ8	0.76 ± 0.2[a]	0.35 ± 0.1[b]
	20:4n-6	11.2 ± 1.8[a]	7.6 ± 1.4[b]
	22:4n-6	1.4 ± 0.9	1.5 ± 0.3
	Total n-6 PUFA	27.6 ± 3.2[a]	17.9 ± 2.6[b]
n-3 Polyunsaturated fatty acids			
	18:3n-3	0.18 ± 0.1	0.15 ± 0.1
	20:5n-3	0.37 ± 0.2[b]	1.8 ± 0.3[a]
	22:5n-3	0.56 ± 0.3[a]	3.4 ± 0.7[b]
	22:6n-3	1.3 ± 0.6[b]	8.8 ± 1.3[a]
	Total n-3 PUFA	2.4 ± 0.5[b]	14.2 ± 1.6[a]
	Total PUFA	29.9 ± 3.0	32.2 ± 3.3

All values are expressed as percent of total plasma phospholipid fatty acids (mean ± SD). Rows with different letters are significantly different between experimental diet groups ($P < 0.05$). LALA, low α-linolenic acid diet; DHA, docosahexaenoic acid diet; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

Table 11. Total cholesterol (TC), esterified cholesterol (EC), free cholesterol (FC), and triacylglycerols (TAG) over the 63 day feeding trial.[1]

		LALA	MALA	HALA	DHA
TC					
	Day 0	476.9 ± 86.5	393.7 ± 85.4	377.3 ± 65.7	496.5 ± 105.5
	Day 28	420.0 ± 75.9	350.6 ± 72.5	341.5 ± 46.6	379.8 ± 61.0
	Day 63	352.2 ± 38.5	nd[2]	nd	359.0 ± 142.7
EC					
	Day 0	371.2 ± 62.7	308.4 ± 58.0	296.2 ± 47.5	385.5 ± 76.9
	Day 28	299.7 ± 54.1	252.3 ± 47.2	246.4 ± 32.0	271.9 ± 44.6
	Day 63	212.3 ± 36.8	nd	nd	216.3 ± 84.9
FC					
	Day 0	105.8 ± 25.5	85.3 ± 30.4	81.0 ± 10.4	111.0 ± 29.3
	Day 28	120.2 ± 22.6	98.3 ± 25.7	95.1 ± 15.4	107.9 ± 19.1
	Day 63	139.9 ± 14.2	nd	nd	142.7 ± 58.3
TG					
	Day 0	115.0 ± 14.1	118.7 ± 31.3	107.6 ± 16.3	107.1 ± 21.2
	Day 28	132.6 ± 13.5	125.0 ± 28.6	123.3 ± 26.6	133.9 ± 32.8
	Day 63	176.9 ± 30.9	nd	nd	191.0 ± 109.4

[1]Values are expressed in mg/dL (mean ± SD). LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet.
[2]nd, not determined.

Table 12. Erythrocyte lysate malondialdehyde (MDA), catalase (CAT), and superoxide dismutase (SOD) normalized with erythrocyte lysate protein over the 63 day feeding trial.[1]

		LALA	MALA	HALA	DHA
MDA (μmol/g protein)					
	Day 0	0.015 ± 0.006	0.012 ± 0.003	0.011 ± 0.002	0.009 ± 0.002
	Day 28	0.015 ± 0.003	0.014 ± 0.003	0.014 ± 0.002	0.015 ± 0.003
	Day 63	0.04 ± 0.01	nd[2]	nd	0.05 ± 0.03
CAT (nmol/min/mg protein)					
	Day 0	134.7 ± 48.2	109.6 ± 37.0	151.6 ± 38.8*	83.7 ± 22.8
	Day 28	109.0 ± 23.8	116.5 ± 31.0	121.9 ± 43.6	132.6 ± 65.5
	Day 63	82.5 ± 23.2	nd	nd	106.0 ± 34.3
SOD (U/mg protein)					
	Day 0	1.7 ± 0.4	2.2 ± 0.6	2.1 ± 0.8	1.8 ± 0.7
	Day 28	1.1 ± 0.3	1.4 ± 0.3	1.2 ± 0.3	1.1 ± 0.3
	Day 63	0.8 ± 0.2	nd	nd	0.9 ± 0.3

[1]Values are expressed as mean ± SD. LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet. Rows with an asterisk are significantly different from the DHA group.
[2]nd, not determined.

Discussion

The reason for the time effect observed for feed intake over the first 4 weeks is unknown. It could be due to normal eating variability or residual effects of transitioning to a new diet. Lumeij and colleagues (47) observed limited to moderate feed intake for the first four days after abruptly changing from a seed to an extruded diet in various parrot species. For day 63, the increase in ambient temperature that occurred towards the end of the study likely resulted in reduced feed intake for all experimental groups. Since

all the experimental diets were isocaloric, the increase in feed intake with no effect on body weight observed for the DHA group suggests Monk parrots did not utilize the DHA diet as efficiently as the other experimental diets. However, high feed efficiency is not a primary goal in pet bird nutrition.

The time effect on body weight may also have been due to the dietary transition during the acclimation period. It was observed that the birds gained weight from week -6 until week -3, then subsequently lost weight until day 0. It may be possible that the birds create excess fat stores during a diet transition in order to be prepared when their normal feed source is no longer available. This could be a naturally occurring behavior which would benefit birds during natural changes in available feed sources due to seasons or migration. Parrots abruptly changed from a seed to an extruded diet had a moderate decrease in body weight which was regained within two weeks (47).

The higher plasma phospholipid 16:0 at day 28 in the DHA group may be due to an increase in de novo fatty acid synthesis with this diet. One reason for this possibility is that an increase in saturated fatty acids might be necessary to maintain the overall polyunsaturated (PUFA):saturated fatty acid (SFA) ratio of the plasma phospholipids. This ratio plays a role in the flexibility, fluidity, and function of the cell membrane (reviewed by 71). Platelet fluidity increased when rats and humans were fed high PUFA diets (31). Also, the PUFA EPA, DPA, and DHA substituted for LA, 20:2n-6, and AA in the plasma phospholipids of the DHA group. Lands (72) has developed equations in rats relating dietary lipid composition with predicted phospholipid composition based on the substitution pattern of ALA and its long-chain derivatives when replacing LA in the diet.

Hwang (73) determined a dietary ALA:LA ratio as low as 0.28 (or as high as 3.6) suppressed the incorporation of AA into plasma phospholipids in rats. Similar substitutions have also been observed in chickens (74).

The combination of increased 18:0 with decreased 18:1n-9 in the MALA and HALA groups at day 28 suggests some suppression of stearoyl-CoA (Δ-9) desaturase (SCD). Previous studies have observed such suppression to exist at high dietary PUFA (75, 76). Velliquette (77) has shown that LA and EPA, but not ALA directly, can down regulate SCD transcription in humans. When comparing n-3 and n-6 PUFA, SCD expression was decreased in bulls fed a diet higher in ALA compared to a diet higher in LA (78). However, no effect on SCD activity was seen in pigs on a diet containing 4.2% extruded linseed oil compared to pigs on a diet containing 1.6% sunflower oil (79). Stearoyl-CoA desaturase may have been similarly suppressed in the DHA group, which showed both 18:0 and 18:1n-9 to be decreased. However, in the case of the DHA group, the lower 18:0 may have simply been the result of decreased dietary 18:0, thereby providing less substrate for stearoyl-CoA desaturase.

Plasma phospholipid LA gradually increased as dietary en% ALA increased (Figure 7). This phenomenon suggests a sparing effect on the conversion of LA to ALA. A similar accumulation of serum phospholipid LA was observed in dogs fed 3% “as-fed” whole ground flaxseeds compared to dogs fed 3% “as-fed” whole ground sunflower seeds (80). Rees and colleagues (80) additionally found decreased AA and 22:4n-6 in the flaxseed fed dogs, and suggested dietary ALA reduced the conversion of LA to its long chain derivatives producing the observed sparing effect.

However, a decrease in LA in the DHA group was observed at days 28 and 63 despite equivalent dietary LA in the experimental diets (Table 7 and 13). This suppression of n-6 fatty acids also included 20:2n-6 and AA. One possible explanation is that the n-6 fatty acids are providing metabolic energy to a greater extent rather than being incorporated into plasma phospholipids. The higher 22:4n-6 compared to the MALA and HALA groups, but not the LALA group, is likely due to its inclusion in the diet rather than conversion from LA.

While no difference in LA was observed between the three ALA groups, an increase in 20:2n-6 in the MALA and HALA groups compared to the DHA group occurred, although this fatty acid was lower compared to the LALA group. These findings combined with decreased AA and 22:4n-6 in the MALA and HALA groups suggests some chain elongation of LA to 20:2n-6, but without its further conversion to AA. Trevizan and colleagues (81) observed accumulation of plasma phospholipid and erythrocyte membrane 20:2n-6 (but not AA) in cats fed a high LA diet, which was attributed to saturation of the elongase enzyme with the substrate LA combined with low Δ6-desaturase activity. It is important to note that the MALA, HALA, and DHA diets all suppressed plasma phospholipid AA. Thus less substrate is likely available for conversion to the more pro-inflammatory n-6 derived eicosanoids. Reducing the n-6 derived eicosanoids should lead to a reduction in the pro-inflammatory state of the body, which could aid in preventing the development of atherosclerosis and other inflammatory diseases (23, 24).

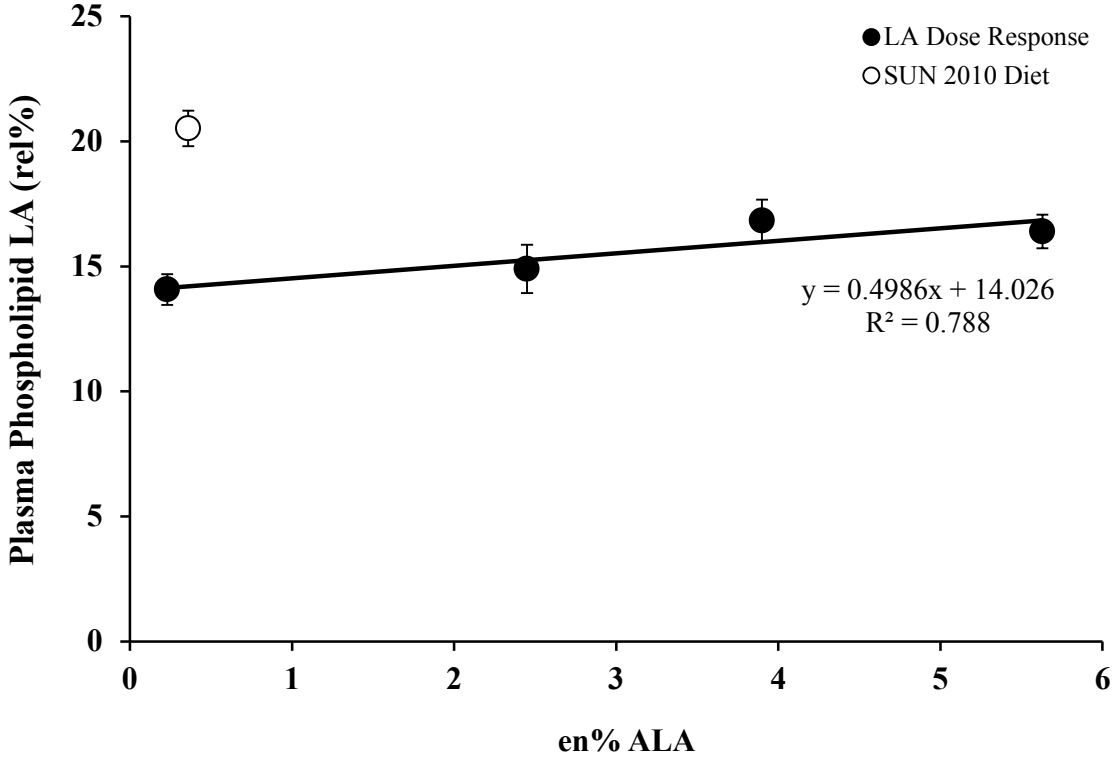

Figure 7. Dose response of plasma phospholipid linoleic acid (LA) at day 28 to increasing dietary en% α-linolenic acid (ALA).
Values are expressed as mean ± SEM. SUN, sunflower seed diet (Chapter II).

Table 13. Amount (en%) of α-linoleic acid (ALA) and linoleic acid (LA) in the experimental diets from study 1 (Chapter II) and 2 (this study).

	SUN	FLX	LALA	MALA	HALA	DHA
ALA (en%)	0.36	5.6	0.23	2.5	3.9	0.16
LA (en%)	8.1	4.4	4.8	5.6	5.5	4.5
LA:ALA	22.5	0.8	20.9	2.2	1.4	28.1

SUN, sunflower seed diet; FLX, flaxseed diet; LALA, low LALA, low α-linolenic acid diet; MALA, medium α-linolenic acid diet; HALA, high α-linolenic acid diet; DHA, docosahexaenoic acid diet.

The increase of 22:4n-6 observed in the LALA group may also be due to elongation of AA. It is possible that when AA reaches a certain level, it begins being converted to 22:4n-6 to prevent AA levels from increasing further. Accumulation of neutrophil phospholipid 22:4n-6 was observed in dogs fed diets containing safflower oil or beef tallow (82). Waldron and colleagues (82) hypothesized that accumulation of 22:4n-6 served as a reservoir for AA upon retro-conversion.

It is even more noteworthy that birds in all of the ALA experimental groups accumulated DHA in their plasma phospholipids. This finding is important because none of the ALA diets contained DHA (nor EPA or DPA), thus the birds appeared to readily convert ALA to DHA, unlike most mammals studied to date (21). Furthermore, although the DHA diet also contained DPA, little ALA and no EPA was present. Nonetheless, the DHA group had the same amount of EPA as the MALA group (2.5 en% ALA). This suggests Monk parrots are also capable of retro-converting DPA/DHA to EPA. Gronn (83) found 20% of ^{3}H-labelled DHA was retro-converted to EPA in rat hepatocytes in vitro. The retro-conversion rate was not affected by (-)-carnitine or (+)-decanoylcarnitine suggesting retro-conversion of DHA is a peroxisomal function rather than a mitochondrial function (83). When 50mg ^{13}C-DHA was given to elderly and young adult humans, mainly ^{13}C-EPA was observed 24 hours after dosing in both age groups (84). However, it is important to note that Plourde and colleagues (84) also observed ^{13}C-DPA and ^{13}C-ALA. Brossard and colleagues (85) found in vivo retro-conversion of ^{13}C-DHA to ^{13}C-DPA and ^{13}C-EPA to be 9% in rats, but only 1.4% in humans. Brossard and

colleagues (85) suggested retro-conversion of DHA occurs by one cycle of ß-oxidation followed by reduction to form EPA, with DPA being formed by elongation of EPA.

Dietary ALA was reflected in its accretion into the plasma phospholipid fraction. In addition, the increased amounts of EPA and DPA in the MALA and HALA groups suggests conversion of ALA to both EPA and DPA as dietary ALA concentrations increased and appears to be dose related.

For that matter, a dose response to dietary ALA was observed for plasma phospholipid ALA, with values generally increasing as dietary ALA increased (Figure 8). However, a third order polynomial line fit the data more closely than a linear line. Upon inspecting the graph (Figure 8), an inflection point appears to occur at approximately 3.2 en% ALA. After dietary inclusion levels exceeded this inflection point, plasma phospholipid ALA began to further accumulate in the phospholipids.

As dietary en% ALA increased, plasma phospholipid AA decreased and EPA increased (Figure 9). Plasma phospholipid AA appeared to start leveling out at a dietary inclusion level of 5.6 en% ALA. Similar to the ALA curve, a third order polynomial appeared to be the best fit for the plasma phospholipid EPA data and the inflection point again appeared to be around 3.2 en% ALA.

It may be possible that the inflection points observed for ALA and EPA were the result of a high enough tissue accretion of ALA to reach the Km for the Δ6-desaturase thereby supporting an increased rate of conversion thereafter. In dogs, the Δ6-desaturase Km for ALA substrate was lower than that for LA substrate, but a much higher Δ6-desaturase Vmax for ALA substrate existed (20).

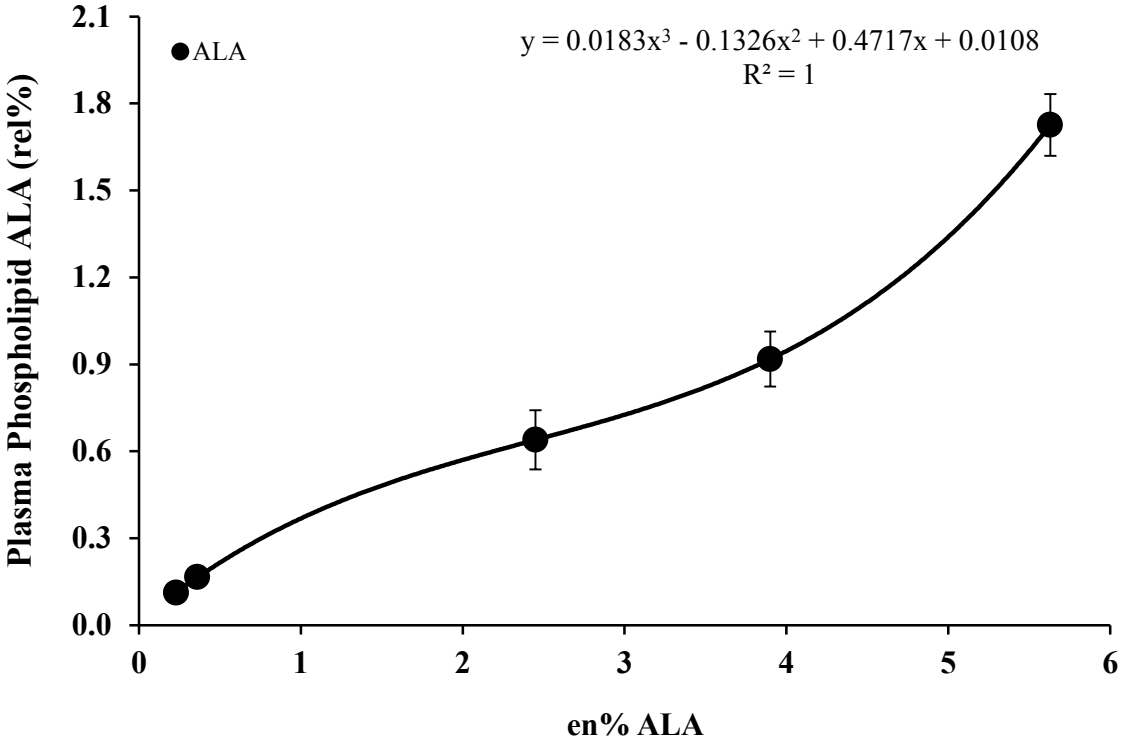

Figure 8. Dose response of plasma phospholipid α-linolenic acid (ALA) at day 28 to increasing dietary en% ALA. Values are expressed as mean ± SEM.

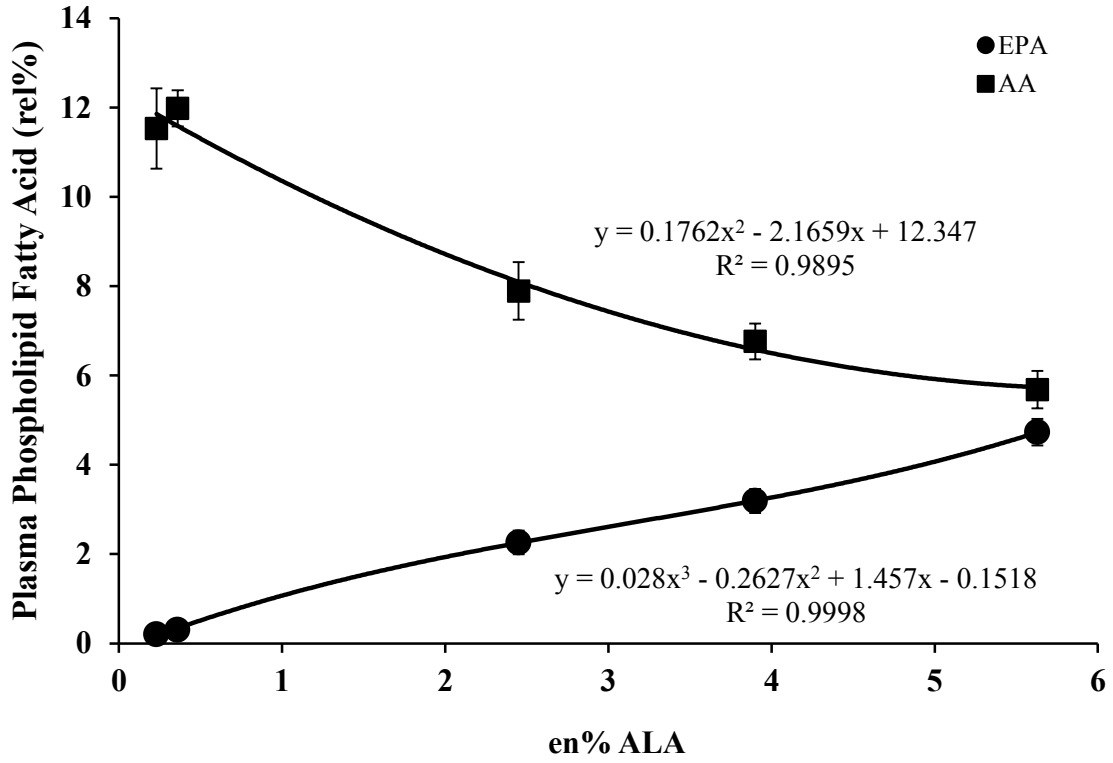

Figure 9. Dose response of plasma phospholipid arachidonic acid (AA) and eicosapentaenoic acid (EPA) at day 28 to increasing dietary en% α-linolenic acid (ALA). Values are expressed as mean ± SEM.

A higher Δ6-desaturase Vmax for ALA substrate was also observed in rats (20). Thus, once the Δ6-desaturase Km for ALA substrate was reached, ALA would be desaturated much more rapidly than LA. Should a similar relationship exist in Monk parrots, it might help explain why increased EPA accumulation was observed once dietary inclusion levels of ALA surpassed 3.2 en%. This is especially the case because all EPA present in the plasma phospholipids was produced from conversion of ALA as EPA was not provided in the diet. The slower desaturation of LA may also explain, in part, the decreased levels of AA in the plasma phospholipid as en% ALA of the diet increased. It

is also likely that the incorporation rate of AA and EPA into the plasma phospholipids was altered. It is important to note that the dietary en% LA remained fairly constant (with the exception of the high LA sunflower seed diet from Chapter II) for all the ALA diets. In order to exceed the inflection point observed at 3.2 en% ALA, which fell between the values found in the MALA (2.5 en% ALA) and HALA (3.9 en% ALA) diets, diets may need to also maintain a LA:ALA ratio between 2.2 and 1.4 (Table 13).

In addition to a possible increase in ALA being converted to EPA after dietary inclusion of 3.2 en% ALA, ALA also continued to accumulate in the plasma phospholipids. The further accumulation of ALA in the plasma phospholipids after the Km for delta-6 desaturase was probably exceeded is likely due to the overall coordinate regulation of the fatty acid pool. α-Linolenic acid has been shown to be a preferred substrate over LA for ß-oxidation (86, 87). An increase in 3-hydroxy-acyl-CoA dehydrogenase and citrate synthase, but not carnitine palmitoyltransferase-I, was observed in mice fed high fat, high n-6 or n-3 PUFA diets compared to a low fat, low PUFA diet (26). Fatty acid synthase and acetyl-CoA carboxylase were decreased in mice fed a high fat, high n-6 PUFA diet, but not in mice fed a high fat, high n-3 PUFA diet or a low fat, low PUFA diet (26). These alterations suggest an overall increase in β-oxidation with no change in fatty acid biosynthesis occurs when mice are fed a high fat, high n-3 PUFA diet. If similar alterations occur in Monk parrots, then an increase in β-oxidation may have occurred, but the preferred substrate likely shifted from ALA to LA, since ALA continued to accumulate in the plasma phospholipids.

A continuous accumulation of DPA was the expected result for this study, because elongation of EPA to DPA readily occurs. Additionally, research in mammals typically observe a low conversion rate of DPA to DHA (21), suggesting DPA would accumulate faster than it could be converted to DHA. However, plasma phospholipid DPA only accumulated until approximately 3 en% ALA. After this point, it gradually decreased and appeared to begin leveling off around a dietary inclusion level of 5 en% ALA (Figure 10). The decrease in plasma phospholipid DPA may have been due to its continued conversion to DHA, especially since plasma phospholipid DHA continuously accumulated as dietary en% ALA increased (Figure 10). It is also possible that the specificity for DPA was altered above 3 en% ALA in the diet. While a linear line best fit the data, the plasma phospholipid DHA levels showed some signs of reaching a plateau when fed higher en% ALA. However, this was not definitively established in this study. The inclusion of 3 en% ALA in the diet again appeared to demonstrate a threshold effect some because at this point DPA no longer accumulated and began to be decreased in plasma phospholipids. It is possible that the accumulation of DHA when dietary ALA exceeded 3 en% may have reached a point where some feedback inhibition on its further conversion from DPA occurred, thus favoring retro-conversion to EPA. Consistent with this possibility is an earlier study in growing rats that found the inclusion of preformed dietary DHA decreased the net biosynthesis and/or accretion of deuterium labeled DHA converted from labeled ALA 2 to 5 fold compared to growing rats with no preformed DHA in their diet (88). Enzyme specificity for DPA may have also decreased when dietary ALA exceeded 3 en%. In contrast to the ALA diets, the DHA diet resulted in

plasma DHA levels that were significantly higher than that amount found with the HALA diet. Thus, directly feeding DHA appeared to increase plasma DHA levels above those that were obtained by feeding only ALA as an n-3 fatty acid source. The HALA diet contained 1.4 g ALA/100 g diet while the DHA diet contained 1.6 g DHA/100 g diet and only 0.06 g ALA/100 g diet. Substituting a similar amount of dietary DHA for ALA can provide larger plasma phospholipid accumulations of DHA in Monk parrots. Thus the same amount of dietary n-3 may provide more beneficial effects for reducing inflammatory diseases, such as atherosclerosis, when fed as DHA rather than ALA.

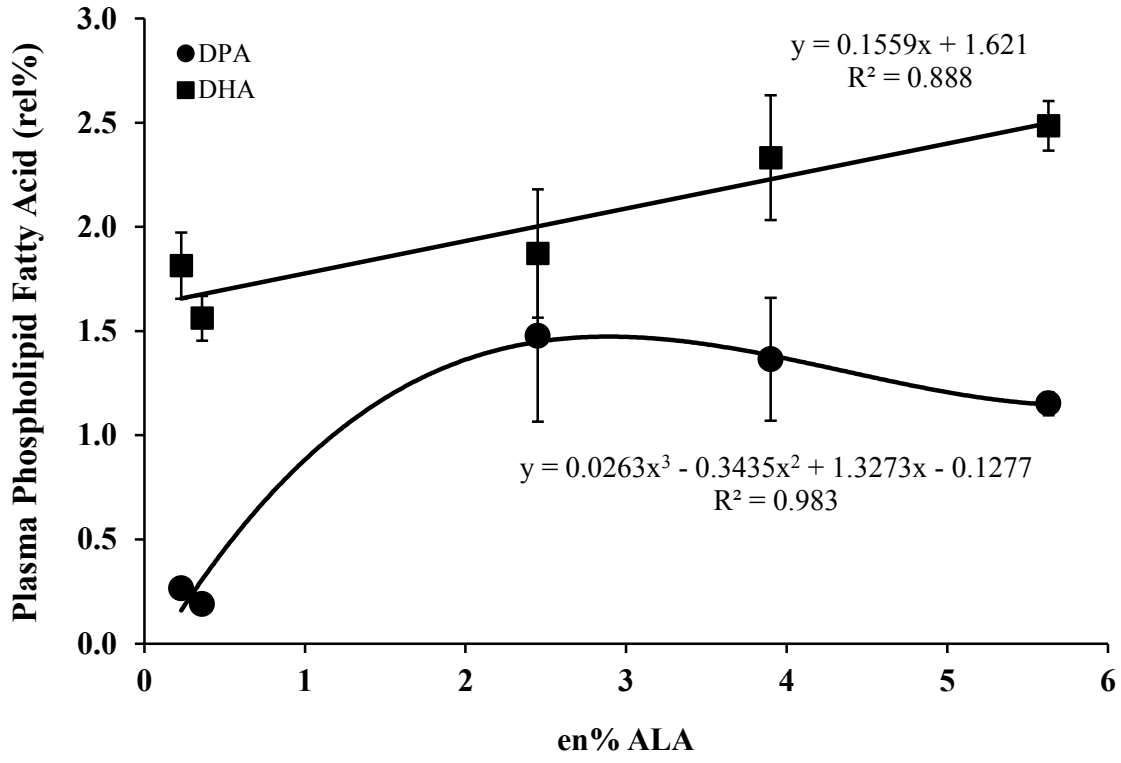

Figure 10. Dose response of plasma phospholipid docosapentaenoic (DPA) and docosahexaenoic acid (DHA) at day 28 to increasing dietary en% α-linolenic acid (ALA). Values are expressed as mean ± SEM.

Dose responses to dietary LA, ALA, EPA and DHA on fatty acid adipose and intramuscular fat composition in pigs was analyzed by Nguyen (89). Unlike this study, a strong linear response to dietary ALA was observed for adipose ALA composition in the pigs. However, comparison between the two studies is difficult as 3 out of the 6 diets fed to the pigs also contained fish oil. Additionally the composition of plasma phospholipids was analyzed in the present study, while adipose tissue composition was analyzed in the pigs (89).

The reason for the decrease in TC with time, regardless of experimental diet, is unknown at this time. Total cholesterol was observed to change markedly over the course of this study as well as variable increases as observed in Chapter 2. It is unknown whether other physiological determinants have a more prominent role in regulating plasma TC in Monk parrots than the diets fed. It may be that ambient temperature, environmental lighting, breeding season, molt, stress, *de novo* fatty acid synthesis, and/or other hormonal influences are also important regulators of plasma TC in Monk parrots. With the decrease in TC, EC also decreased while FC modestly increased. Plasma TAG also increased over the study period regardless of diet. In Study 1 (Chapter II), plasma TAG was decreased when birds were fed the acclimation diet, which contained 4% fat, and increased after the birds were switched to the higher fat experimental diets (5.7-6.1% fat). However, the acclimation diet for this study was also higher in fat (6.5%) than their normal maintenance diet when not on studies (4.2% fat). Thus, the increase in TAG may be the result of increasing fat in the diets. The acclimation diet from Study 1 also had a lower PUFA:SFA ratio than Zupreem Natural®

and the experimental diets. However, in this feeding trial there was a great variation in dietary PUFA:SFA ratios (Table 7), but no significant dietary differences were observed. This suggests that the PUFA:SFA ratio was not the cause for the change in TAG over time observed in Study 1. Humans fed a corn oil or soybean oil containing diet had significantly higher total cholesterol than those consuming a sunflower oil containing diet after 5 months (90). Javadi and colleagues (26) observed a decrease in plasma TAG concentrations in mice fed high fat, high n-3 or n-6 PUFA diets compared to a low fat, low PUFA diet. Plasma TC concentrations were also decreased for the high fat, high n-3 PUFA diet (26). Newman and colleagues (91) also observed decreased plasma TAG concentrations in chickens fed a diet containing fish oil compared to tallow. Plasma TAG levels for the sunflower oil diet in the latter study did not differ from either the fish oil diet or the tallow diet (91). However, there were no diet differences observed by Newman et al. (91) in plasma TC or FC concentrations in the chickens. The observations by Newman and colleagues (91) combined with those for this study suggests that dietary PUFA do not similarly alter plasma cholesterol or TAG concentrations in avian species as in mammals. Thus, although cholesterol and TAG are good risk factors for atherosclerosis in mammals, this may not be the case in avians.

The erythrocyte samples were stored at -80°C ranging over a period of 3 to 38 days prior to analyzing MDA. Thus, the effect of storage time on MDA values was assessed (results not shown). There was a slight, but not significant, increase in MDA when the erythrocyte samples were stored at -80°C rather than analyzed freshly after collection. A significant increase in MDA occurred after storing the samples at -80°C for

6 weeks, so storage did not affect the MDA values of the samples in this study because all samples were analyzed within 38 days of storage.

It should be noted that as SOD values decreased, MDA values increased. This suggests that SOD plays some role in removing MDA from the plasma of Monk parrots. While these findings may also suggest an increase in *in vivo* oxidation, there are many oxidative species and antioxidant enzymes, which may affect the overall oxidative state, that were not measured in this study. Additionally, the validity of MDA as a measurement of peroxidation has been questioned (92). Iraz and colleagues (67) supplemented rats with long chain n-3 fatty acids and found no difference in SOD compared to placebo-supplemented rats. Increased CAT activity and decreased MDA were also observed in rats supplemented with long chain n-3 fatty acids. A smaller dosage of long chain n-3 fatty acids, however, were provided to the rats in that study compared to the Monk parrots in the present study (Iraz et al. 1.4 g/kg/day versus our 1.7 g/kg/day). When compared on a mg/day basis, the rats in the Iraz et al. (69) study were supplemented with approximately 120 mg EPA+DHA/day while the Monk parrots received about 196 mg DPA+DHA/day. Krasicka and colleagues (93) observed increased MDA in the breast and leg muscles of chickens fed an 8% FLX diet compared to 0% FLX, 8% rapeseed, or 6% FLX plus 6% rapeseed diet.

Muggli (94) determined amounts of vitamin E required to compensate for PUFA in the diet of mammals including humans. Harris (95) compared studies reporting adequate and inadequate vitamin E:PUFA ratios. For chickens, and most mammals, a vitamin E:PUFA ratio of 0.6 or higher was deemed adequate, while less than 0.6 was

inadequate and resulted in deficiency symptoms (95). All the diets used in this study had vitamin E:PUFA ratios of 1.37 or greater based on estimated vitamin E amounts added to the diets. Based on these estimates, the diets used in this study met or exceeded the vitamin E needs of the Monk parrots. However, the decrease in SOD and increase in MDA suggests the diets did not contain enough vitamin E or other antioxidants. An interesting note is that the flaxseed diet utilized by Krasicka and colleagues (93) resulted in increased MDA in the chickens even though the diet contained a vitamin E supplement greater than 200 mg/kg diet. While this does not fully explain the increased MDA observed for all the Monk parrots in this study despite diet, it is possible that some compound(s) in flaxseed, in addition to its n-3 content, promote oxidation and radical formation. Additionally, Todd et al. (96) found that high fat diets stimulate fatty acid oxidation more than low fat diets. However, it is unknown if antioxidants, especially vitamin E, were increased in the high fat diets used in their study.

CHAPTER IV

EFFECT OF DOCOSAHEXAENOIC ACID ON LEARNING ABILITY IN MONK PARROTS

Introduction

Docosahexaenoic acid (DHA) is a long-chain n-3 fatty acid that is incorporated into the retina, brain, and other neural tissues (42). DHA deficiency has been shown to reduce motor development in mice (44). The DHA requirements for normal motor development and function in Monk parrots are currently unknown. The lay press recognize these birds as popular pets because of their mimicking skills and strong personalities (97). It is believed that birds experience a sensitive period for learning while young (98). Because DHA plays a role in motor development, especially during growth, this study tested the hypothesis that providing increased pre-formed dietary DHA under known conditions improves the learning ability of young adult Monk parrots.

Materials and Methods

This study utilized 12 Monk parrots (*Myiopsitta monachus*) from the previous study (described in Chapter III). Birds were housed at the Schubot Exotic Bird Health Center at Texas A&M University. Each bird received a physical examination, including body weight and condition score assessment, gender determination, and was permanently identified with a metal leg band prior to starting the study. Birds were maintained in 0.61 m x 1.22 m x 1.83 m (2’ x 4’ x 6’) cages in pairs (except for two

birds). The birds were housed in a covered facility with two mesh walls, which provided protection from inclement weather. In the winter, heavy curtains were secured over the mesh walls and heat lamps were utilized when the temperature fell below 4.4°C (40°F). When the temperature rose above 32.2°C (90°F), a swamp cooler and large wall fans were used to cool the aviary. Birds were provided feed and water *ad libitum*. The birds underwent a 6 week acclimation period to transition from an extruded to a pelleted diet. They were then randomly assigned to one of two treatment groups (low ALA diet (LALA) or high DHA diet (DHA)) and matched for age within each group. Due to the death of a bird assigned to the DHA group during the diet acclimation period from causes unrelated to the study on day -2, 6 birds in the LALA group and 5 birds in the DHA group completed the study. The LALA group had three 1 year old and three 3-4 year old birds. The DHA group had three 1 year old and two 3-4 year old birds at the time of the study.

Two training cages measuring 0.61 m x 1.22 m x 1.83 m (2’ x 4’ x 6’) with visual isolation and limited tactile interactions were placed in the room where the birds were housed. Visual isolation for each training cage was accomplished by hanging canvas fabric around, but not touching, the training cages. Training cages were similar to the cages in which birds were usually maintained, but did not contain any toys or enrichment items in order to limit tactile interactions and distractions. The training cages did contain two natural wooden perches with bark. Auditory isolation was not achieved during this study. Due to widely varying individual responses to the presence of humans, nobody was present in the training area during training sessions. Thus, all training

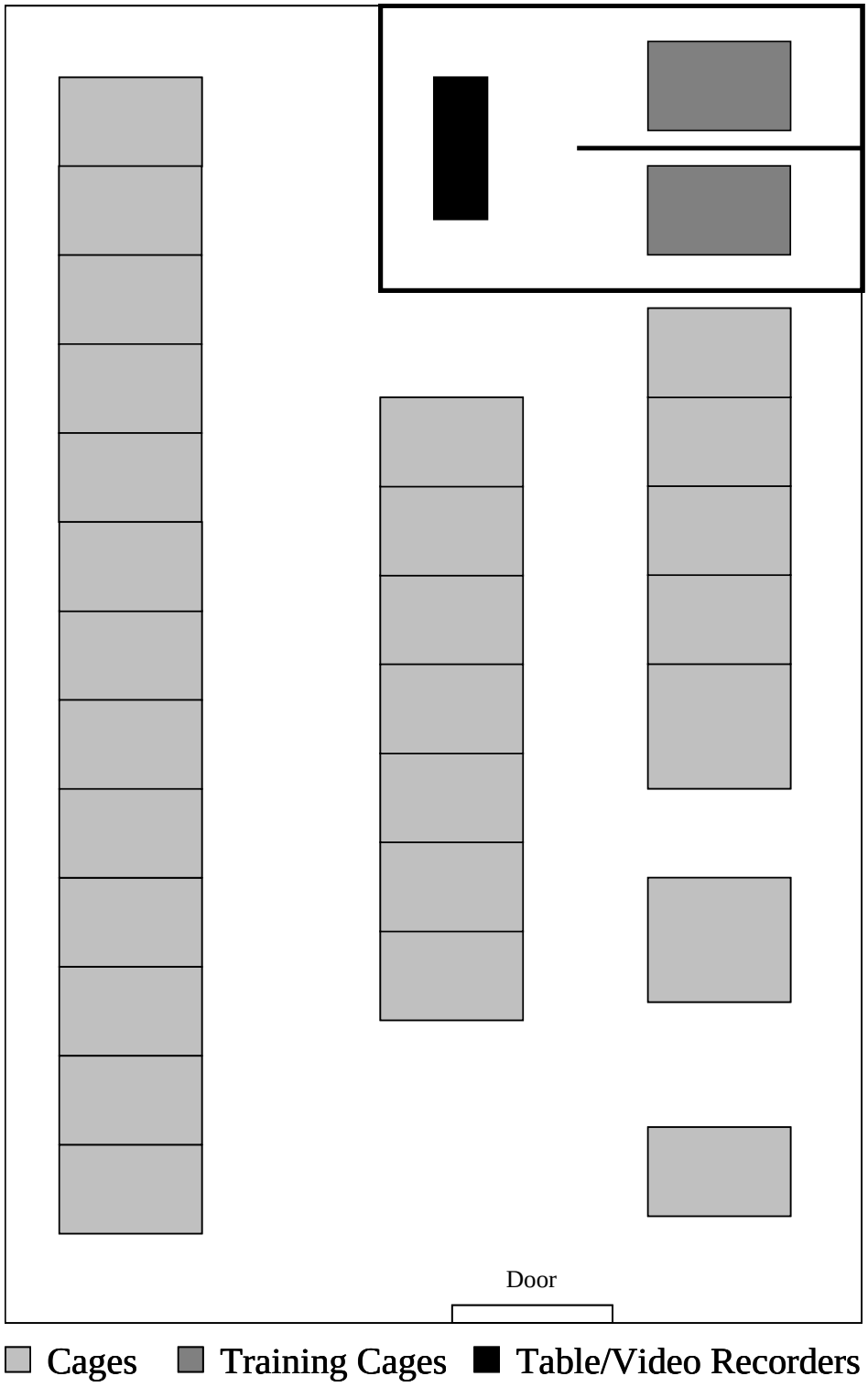

Figure 11. Layout of housing and training cages.

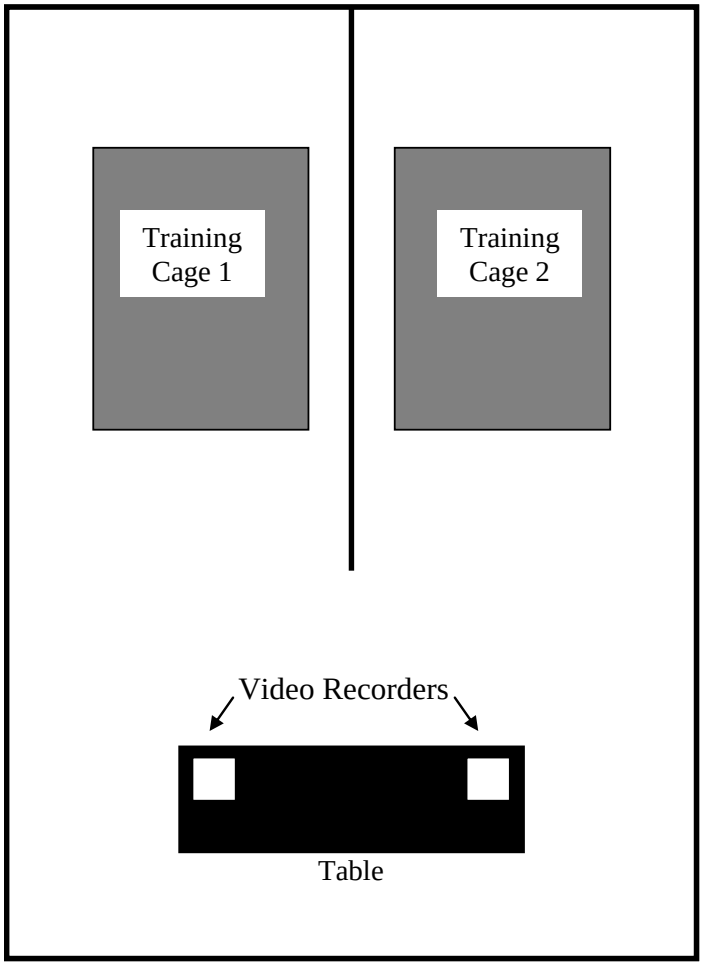

Figure 12. Diagram of training area set-up.
Training cages measured 0.61 m x 1.22 m x 1.83 m. Dark solid lines represent the heavy fabric used to create visual isolation. Video recorders were positioned on the table approximately 0.76 m from the front of the cages.

sessions were video recorded (Flipvideo; Cisco Consumer Products, Irvine, CA) and stored for playback analyses (Windows media player; Microsoft Corporation, Redmond, WA). The recorded area was 0.61 m x 1.22 m x 0.30 m (2’ x 4’ x 1’). A diagram of the room and training area set-up are provided in Figures 11 and 12.

Coloured Cubes® (Zoo-Max Exotic LTD., Quebec, Canada) made from 4 mm thick bird-proof acrylic were used in this study. The Coloured Cubes® measured 44 mm x 53 mm x 44 mm on the outside. The inner empty space when the lid was closed measured 44 mm x 36 mm x 30 mm. The cubes had an 8 mm piece of the lid sticking out past one side of the cube and forming a pull tab. The opposite side of the lid had 6 mm doubled in thickness which prevented the removal of the lid from the cube. The lid of the cube was recessed 6 mm from the top of the cube. Cubes were modified by drilling a hole in the bottom, so they could be secured to the bottom of the training cages. Pieces of plastic cards were also glued to the insides of the cubes to prevent any gaps between the sides of the cube and the lid.

Monk parrots were acclimated to the training cages prior to the first training session. These sessions consisted of placing an individual bird in each training cage for a period of 4 hours which was gradually reduced to 1 hour over a 10 day period. All subsequent sessions consisted of an established amount of time in which the bird had one chance to complete the given task. Session length was based on a set amount of time and not on the bird's accomplishments. Every bird underwent a maximum of 1 session per day during the training periods. Birds had *ad libitum* access to fresh water and the acclimation diet (ACC) while undergoing acclimation to the training cage. Birds were also acclimated to the red colored cube used in the initial training sessions by providing them as an enrichment toy. Acclimation to the red colored cube lasted 24 days (February 28, 2011 until March 23, 2011). Birds were not acclimated to the green or yellow colored cubes, which were used later in this study. Acclimation to the red colored cube

started by placing the cube in the regular feed dish with the ACC diet. Gradually, all the feed was placed in the red colored cube which was then set in the regular feed dish.

During the diet acclimation period, birds successfully completed the Open One Cube learning task (OOC, see below for description of tasks). Following this time, birds were fed their respective experimental diets as described in Chapter III. While fed the experimental diets, the birds underwent one training session per week for 5 weeks in order to maintain the previously learned OOC behavior. For the following 4 weeks, the OOC behavior was extinguished over a total of 13 sessions. The birds, then having consumed the experimental diets for a total of 9 weeks, were subjected to sessions in which they relearned the OOC task (ROOC). After successfully opening the lid in the ROOC task for 3 consecutive sessions, the birds then moved on to the Three Cube Trial (TCT) which contained red, green, and yellow cubes with the feed placed in the green cube. The TCT sessions were stopped after the bird successfully opened the lid to the green cube first in 3 consecutive sessions. If the bird was unsuccessful in completing either the ROOC or TCT task, sessions continued until the experimental diets were depleted 30 days past the first ROOC session.

Learning tasks

Period 1-establishing the open one cube (OOC) behavior

The birds were maintained on the acclimation diet for this task in order to establish baselines for each bird. A red colored cube was secured to the bottom of the cage near where the feed dish was normally placed using a small bolt, 2 washers, and a nut. The water dish was provided as normal; however, no feed dish was placed in the

training cage. Feed was only placed in the red colored cube. The goal of this task was for the bird to open the lid, which would enable access to the feed. To encourage learning the task in a timely manner, the lid was initially opened ¼ for session 1, but was increased to ½ for session 2 before being returned back to ¼ for session 3, and then fully closed for session 4 and thereafter. The lid was again opened ¼ after 3 consecutive unsuccessful sessions. There was no human interaction during the 50 minute sessions. Feed was withheld 2-4 hours prior to training, but this time period was randomized for each bird's individual session.

Period 2-extinguishing the OOC (EOOC) behavior

The set-up for this task was similar to the OOC task above with the exception that no feed was placed inside of the red colored cube. The goal of this task was to document the amount of time necessary for the bird to learn that the feed was no longer present in the cube and thus stop opening the sliding lid. The lid was fully closed for each session. Birds were switched from the acclimation diet to their respective experimental diets prior to beginning the EOOC task. Each session lasted 30 minutes with a total of 13 sessions.

Period 3-relearning the open one cube (ROOC) behavior

This task was identical to the OOC task. However, the birds continued to be fed their respective experimental diets and the sessions remained at 30 minutes in length. The guidelines for lid placement were the same as the OOC task. The bird moved on to the next task once the lid was opened (when starting fully closed) over 3 consecutive

sessions. Training for the unsuccessful birds continued until depletion of the experimental diets (30 days).

Period 4-establishing complex task learning with a three cube trial (TCT)

For this task, three colored cubes (yellow, green, and red) were used. All cubes were constructed from the same type of material and had the same dimensions. Feed was now only placed in the green colored cube, rather than the previously used red colored cube. The additional cubes were secured on either side of the original cube location in a straight line with plenty of space for the bird to walk around each cube. The order of the cubes was changed for each session. This prevented the location of the cubes from providing an effect and ensured the bird would differentiate the cubes by color. All three lids were fully closed for every session. Training sessions remained at 30 minutes in length. Birds stopped being trained after opening the green colored cube lid first on three consecutive training sessions or at the end of the study (30 days from first ROOC session).

Statistical analyses

Data were statistically analyzed for normality with the Shapiro-Wilk test. Diet effects for each task were analyzed using Student's t-tests. Age and OOC learning times were tested as covariates using ANCOVA. Because the data did not meet the linear homogeneity assumption of ANCOVA, the covariates were also tested as variables using two-way ANOVA with Students t-tests for post-hoc analysis as a more fitting non-parametric alternative could not be found. Nonetheless, the results from both methods had similar outcomes. Paired t-tests were also utilized to compare the OOC and ROOC

times to first successful session and three consecutive successful sessions. Prior to analysis, some data were converted into percent of total training time in order to compare groups with animals that completed different numbers of training sessions. In all cases significance was set at $P < 0.05$.

Results

All Monk parrots began eating feed out of the red colored cube within 10-15 minutes after initial exposure to it. Thus no bird appeared extremely afraid of the cube. A total of 7 birds (3 DHA group and 4 LALA group) successfully completed the ROOC task and moved on to the TCT task. Two birds (both LALA group) successfully completed the TCT task three times consecutively. However, 2 more birds in the DHA group had completed only two successful consecutive TCT sessions prior to running out of feed. Thus, the number of birds successfully completing three consecutive TCT sessions may have become the same if the study had been continued for a longer period of time. As such, a total of 46 sessions (average 11.5/bird) were completed by the LALA group during the TCT task training, while 38 (average 12.7/bird) were completed by the DHA group.

When percentage of successfully completed sessions per task were analyzed, no diet effects for any of the learning tasks were observed. Age effects tested using ANOVA resulted in the 1 year old birds having less success than the 3-4 year old birds for the OOC and TCT tasks ($P = 0.005$ and $P = 0.0003$, respectively). The 1 year old birds also tended to have a higher percentage of success for the EOOC task, but it was not significantly different ($P = 0.056$).

Table 14. Times to complete first successful session and third consecutive successful session.

		LALA (n=6)	DHA (n=5)	1 Year Olds (n=6)	3-4 Year Olds (n=5)
Time to 1st successful completion					
	OOC	16,537 ± 3,501	17,196 ± 6,496	22,204 ± 3,025*	10,396 ± 745
	EOOC	7,941 ± 2,847	8,224 ± 2,802	5,314 ± 2,245	11,375 ± 2,730
	ROOC	7,605 ± 1,298	13,120 ± 2,894	12,191 ± 2,523	7,618 ± 3,095
	TCT	17,072 ± 5,440	12,187 ± 8,279	27,125 ± 775*	5,868 ± 1,555
Time to 3rd Consecutive Successful Session					
	OOC	20,435 ± 2,299	21,642 ± 3,166	24,878 ± 2,201*	16,310 ± 932
	EOOC	15,080 ± 3,009	14,306 ± 3,009	10,589 ± 2,071*	19,695 ± 2,900
	ROOC	19,276 ± 5,764	24,368 ± 6,413	24,933 ± 6,418	17,579 ± 5,035

Values are expressed in seconds as mean ± SEM. Rows with an asterisk are significantly different ($P < 0.05$) from the 3-4 year old birds. LALA, low α-linolenic acid diet; DHA, docosahexaenoic acid diet; OOC, open one cube task; EOOC, extinguish open one cube task; ROOC, relearn open one cube task; TCT, three cube trial.

Training times to milestones

The percentage of total training time from the bird's first contact with the cube to that same cube being opened was compared between the two diet groups by learning task (Table 14). There were no significant differences between the two diet groups for OOC, EOOC, or ROOC tasks. When analyzed by ANOVA, an age effect with the 1 year old birds taking significantly more time than the 3-4 year old birds existed for the OOC and EOOC tasks ($P < 0.0001$ and $P = 0.0004$, respectively). For TCT, the times were compared by order of cube opened regardless of color (1st cube opened, 2nd cube opened, or 3rd cube opened) and also by color of cube (regardless of order). The DHA group tended to take a lesser percentage of time than the LALA group to open the 1st cube, though it was not significantly different ($P = 0.05$). No significant differences between the two diet groups existed for the 2nd or 3rd cubes opened. Using ANOVA, the 1 year old birds took significantly more time than the 3-4 year old birds to open the 1st cube ($P < 0.0001$) and 2nd cube ($P < 0.0001$). No diet effects were observed when percent of total training time from first contact with the cube to that same cube being opened was analyzed by color of the cube rather than by order it was opened without considering covariates. After adjusting for age as a covariate, the DHA group took significantly more time from first contact to opening the green cube ($P = 0.02$). By ANOVA, the 1 year old birds took significantly more time than the 3-4 year old birds to open the red and green cubes ($P = 0.0004$ and $P < 0.0001$, respectively).

The total training time in seconds to successfully complete each task one time was analyzed. There were no diet effects for any of the learning tasks. However, there

was an age effect observed for the OOC and TCT tasks. The 1 year old birds took a significantly longer amount of time than the 3-4 year old birds to successfully complete the task one time ($P = 0.01$ and $P = 0.0002$, respectively).

A paired t-test was performed to compare the time needed to successfully complete the OOC task with that of the ROOC task. Overall, the birds took significantly more time to successfully complete the OOC task (16,836 seconds) initially than the ROOC task (10,112 seconds, $P = 0.007$). No significant diet effects were observed between these two tasks. The 1 year old birds tended to take less time to complete the ROOC task when paired with the OOC task by mean difference, though it was not significant (10,013 seconds less time for ROOC for the 1 year olds vs. 2,778 seconds less time for ROOC for the 3-4 year olds, $P = 0.066$). However, the 1 year old birds had a significantly larger mean mean value than the 3-4 year old birds (17,197 and 9,007 seconds, respectively, $P = 0.02$).

The total amount of training time to successfully complete each task three times in a row was also analyzed (Table 14). This analysis could not be completed for the TCT because only two birds successfully completed the task three consecutive times. No diet effects were observed for the OOC, EOOC, or ROOC learning tasks. The 1 year old birds were found to take a significantly longer time learning the OOC task ($P = 0.01$) and a significantly shorter time extinguishing the OOC behavior ($P < 0.04$) than the 3-4 year old birds. A paired t-test was performed to compare the times to the third consecutive successfully completed session for the OOC task with that of the ROOC task, but significant diet and age differences were not observed.

Table 15. Contact times with the cube(s) for each task.

	LALA (n=6)	DHA (n=5)	1 Year Olds (n=6)	3-4 Year Olds (n=5)
Initial contact				
OOC	13.6 ± 3.5	12.8 ± 3.4	17.3 ± 3.7	8.6 ± 2.8
EOOC	6.8 ± 2.2	5.8 ± 2.2	4.2 ± 1.8	8.9 ± 2.6
ROOC	8.8 ± 2.5	8.9 ± 2.6	10.0 ± 2.4	7.0 ± 2.6
TCT 1st Cube	2.0 ± 1.5†	15.1 ± 4.1	6.4 ± 2.9	9.6 ± 3.1
TCT 2nd Cube	7.6 ± 2.9	4.6 ± 1.4	4.6 ± 0.7*	12.0 ± 3.3
TCT 3rd Cube	12.2 ± 4.1	3.8 ± 2.0	1.4 ± 1.4*	15.7 ± 4.5
TCT Red Cube	7.5 ± 3.0	8.4 ± 2.9	4.8 ± 2.5	11.1 ± 3.4
TCT Green Cube	2.4 ± 1.5†	12.6 ± 3.7	3.1 ± 2.1*	11.1 ± 3.2
TCT Yellow Cube	12.0 ± 4.1†	2.8 ± 1.4	0.8 ± 0.7*	15.3 ± 4.5
Before cube opened				
OOC	1.3 ± 0.3	2.4 ± 0.8	2.3 ± 0.7	1.4 ± 0.3
EOOC	0.3 ± 0.1†	0.7 ± 0.2	0.4 ± 0.1	0.5 ± 0.1
ROOC	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
TCT 1st Cube	0.3 ± 0.1	1.3 ± 0.6	0.07 ± 0.04*	1.5 ± 0.5
TCT 2nd Cube	0.3 ± 0.1	2.1 ± 0.9	0.04 ± 0.03*	2.3 ± 0.9
TCT 3rd Cube	0.09 ± 0.04	0.05 ± 0.03	0.02 ± 0.02*	0.1 ± 0.05
TCT Red Cube	0.3 ± 0.1	1.0 ± 0.4	0.06 ± 0.04*	1.2 ± 0.4
TCT Green Cube	0.3 ± 0.1†	2.2 ± 0.9	0.04 ± 0.03*	2.4 ± 0.9
TCT Yellow Cube	0.09 ± 0.04	0.05 ± 0.03	0.02 ± 0.02	0.1 ± 0.05
After cube opened				
OOC	28.9 ± 4.4	35.8 ± 5.3	15.7 ± 3.9*	51.2 ± 3.8
EOOC	2.0 ± 0.6†	1.1 ± 0.5	0.3 ± 0.2*	3.1 ± 0.8
ROOC	11.6 ± 3.1	15.0 ± 2.8	8.2 ± 1.8*	21.4 ± 4.4
TCT 1st Cube	12.5 ± 4.1	6.2 ± 2.5	0.1 ± 0.1*	19.6 ± 4.7
TCT 2nd Cube	18.2 ± 5.0	7.2 ± 2.5	0.7 ± 0.6*	26.4 ± 5.4
TCT 3rd Cube	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
TCT Red Cube	0.4 ± 0.2	0.3 ± 0.1	0.1 ± 0.1*	0.6 ± 0.2
TCT Green Cube	28.2 ± 5.5†	10.7 ± 3.2	0.6 ± 0.6*	40.9 ± 5.5
TCT Yellow Cube	0.1 ± 0.1	0.004 ± 0.03	0.004 ± 0.02	0.1 ± 0.1
Total contact				
OOC	30.2 ± 4.4	38.2 ± 5.1	18.0 ± 3.8*	52.5 ± 3.7
EOOC	2.2 ± 0.6	1.9 ± 0.5	0.8 ± 0.3*	3.6 ± 0.8
ROOC	11.9 ± 3.1	15.3 ± 2.8	8.5 ± 1.8*	21.8 ± 4.5
TCT 1st Cube	12.8 ± 4.1	7.6 ± 2.6	0.2 ± 0.1*	21.1 ± 4.7
TCT 2nd Cube	18.5 ± 5.0	9.3 ± 2.7	0.7 ± 0.7*	28.7 ± 5.3
TCT 3rd Cube	0.2 ± 0.1	0.05 ± 0.03	0.02 ± 0.02	0.2 ± 0.1
TCT Red Cube	0.7 ± 0.2	1.2 ± 0.4	0.2 ± 0.1*	1.7 ± 0.4
TCT Green Cube	28.5 ± 5.6†	12.9 ± 3.3	0.7 ± 0.7*	43.3 ± 5.3
TCT Yellow Cube	0.2 ± 0.1	0.06 ± 0.03	0.03 ± 0.02	0.2 ± 0.1
TCT All Cubes	29.4 ± 5.7†	14.2 ± 3.5	0.9 ± 0.7*	45.3 ± 5.4

Values are expressed as a percentage of the total session training time (mean ± SEM). Multiple sessions made up each learning task. Rows with an asterisk are significantly different ($P < 0.05$) between age groups, and a cross are significantly different ($P < 0.05$) by diet groups. LALA, low α-linolenic acid diet; DHA, docosahexaenoic acid diet; OOC, open one cube task; EOOC, extinguish open one cube task; ROOC, relearn open one cube task; TCT, three cube trial.

Contact times

The amount of time for each bird to initially come in contact with the cube was analyzed both for time in seconds and as a percentage of total training time for that session (Table 15). Similar results were obtained by both methods. For TCT, the times were compared by order of cube opened (1st cube opened, 2nd cube opened, or 3rd cube opened) regardless of color and also by color of cube (regardless of order). No significant diet effects between the two dietary groups for amount of time to initially come in contact with the cube occurred for the OOC, EOOC, or ROOC tasks. For the TCT, the DHA group took significantly longer than the LALA group to initially touch the green cube ($P < 0.02$), and significantly less time to initially touch the yellow cube ($P < 0.05$). The DHA group took significantly longer to initially touch the 1st cube ($P < 0.01$), and significantly less time to initially come in contact with the 3rd cube ($P = 0.01$). Age was a covariate for OOC task, red cube, green cube, 2nd cube opened, and 3rd cube opened. By ANOVA, the 1 year old birds tended to take longer than the 3-4 year old birds to initially touch the cube during the OOC task, though it was not significant ($P < 0.066$). The 1 year old birds took a significantly smaller percentage of time to initially touch the green cube ($P < 0.04$), yellow cube ($P < 0.05$), 2nd cube opened ($P = 0.0017$), and the 3rd cube opened ($P = 0.004$).

The amount of time between initial contact with the cube and that same cube's lid being opened was analyzed both for time in seconds and as a percentage of total training time for that session (Table 16). For TCT, the times were compared by order of cube opened (1st cube opened, 2nd cube opened, or 3rd cube opened) regardless of color

Table 16. Time from initial contact to the same cube's lid being opened for each task (mean ± SEM).[1]

	LALA (n=6)	DHA (n=5)	1 Year Olds (n=6)	3-4 Year Olds (n=5)
Percentage of training time[2]				
OOC	37.2 ± 7.7	30.2 ± 7.5	52.3 ± 8.0*	12.6 ± 5.1
EOOC	71.0 ± 5.2	65.6 ± 5.8	81.1 ± 4.4*	53.3 ± 6.2
ROOC	57.3 ± 5.9	54.0 ± 6.1	59.0 ± 5.3	50.4 ± 6.9
TCT 1st Cube	59.0 ± 7.3	38.2 ± 7.6	83.9 ± 5.6*	13.7 ± 4.9
TCT 2nd Cube	68.2 ± 6.8†	80.9 ± 6.3	97.0 ± 2.4*	49.8 ± 7.8
TCT 3rd Cube	93.7 ± 3.6†	100.0 ± 0.0	100.0 ± 0.0*	92.9 ± 4.0
TCT Red Cube	68.2 ± 6.8	64.5 ± 7.7	83.9 ± 5.6*	48.4 ± 7.6
TCT Green Cube	61.2 ± 7.2	62.5 ± 7.7	99.3 ± 0.7*	22.4 ± 6.3
TCT Yellow Cube	93.7 ± 3.6	97.4 ± 2.6	97.7 ± 2.3	92.9 ± 4.0
Time in seconds				
OOC	100.4 ± 41.1	152.4 ± 78.5	138.3 ± 92.1	119.7 ± 44.9
EOOC	76.5 ± 60.4	46.5 ± 14.8	45.2 ± 20.5	69.4 ± 44.9
ROOC	59.6 ± 33.0	11.6 ± 4.5	49.8 ± 28.5	15.5 ± 5.9
TCT 1st Cube	15.9 ± 2.7	113.4 ± 57.9	18.5 ± 9.9	81.3 ± 40.0
TCT 2nd Cube	49.7 ± 23.7	182.1 ± 164.6	587.5 ± 581.5*	41.9 ± 17.7
TCT 3rd Cube	57.0 ± 15.3	nd[3]	nd	57.0 ± 15.3
TCT Red Cube	36.7 ± 20.1	37.1 ± 13.3	18.5 ± 9.9	41.9 ± 14.8
TCT Green Cube	16.0 ± 3.7	109.7 ± 83.0	173.0 (1 obs)	58.2 ± 41.6
TCT Yellow Cube	57.0 ± 15.3	6.0 (1 obs)[4]	6.0 (1 obs)	57.0 ± 15.3

[1]Rows with an asterisk are significantly different ($P < 0.05$) between age groups, and a cross are significantly different ($P < 0.05$) by diet groups. LALA, low α-linolenic acid diet; DHA, docosahexaenoic acid diet; OOC, open one cube task; EOOC, extinguish open one cube task; ROOC, relearn open one cube task; TCT, three cube trial.
[2]Values are expressed as a percentage of the total session training time (mean ± SEM).
[3]nd, no birds open the cube.
[4](1 obs), only 1 bird opened the cube for a total of 1 session, thus no SEM could be calculated.

and also by color of cube (regardless of order).There were no diet effects observed when analyzed in seconds. The 1 year old birds took significantly more time between initial contact with the cube and opening that same cube's lid for the 2nd cube opened in the TCT task. Since the times expressed in seconds lowered the number of available observations due to it not accounting for the times birds did not open the cubes, the data were also analyzed as a percentage of total training time for that session. When expressed as a percentage, the DHA group spent significantly more time between initial contact and opening the same cube's lid for the 2nd and 3rd cube opened in the TCT task. No other diet effects were observed. An age effect with the 1 year old birds taking a significantly larger percentage of total training time for the OOC and EOOC tasks was observed. The 1 year old birds also took a larger percentage of time for the 1st cube opened, 2nd cube opened, 3rd cube opened, red cube, and green cube in the TCT tasks.

The amount of time the bird interacted with the cube prior to the cube being opened; after the cube was opened; and total amount of time interacting with the cube for each session were also analyzed for both time in seconds and percentage of total training time for that session for all learning tasks (Table 15). Similar results were obtained for time and percentage data. For the TCT data, the times and percentages were compared by order of cube opened regardless of color (1st cube opened, 2nd cube opened, or 3rd cube opened) and also by color of cube (regardless of order). For the EOOC task, the DHA group had significantly more contact with the cube prior to the lid being opened than the LALA group ($P < 0.05$). For the TCT task, the DHA group had significantly more contact with the green cube prior to the lid being opened ($P < 0.05$),

and significantly less after the lid was opened ($P < 0.01$). The DHA group also had significantly less total contact time with the green cube over the entire session than the LALA group ($P < 0.02$). Additionally, the DHA group had significantly less total contact time for the sum of all three cubes over the entire session compared to the LALA group ($P < 0.03$). No significant diet effects for the order the cubes were opened were found without taking covariates into consideration. After adjusting for age as a covariate, a diet effect now existed for the amount of contact time after the cube was opened for the 1st cube (DHA less, $P = 0.02$), 2nd cube (DHA less, $P = 0.0006$), and nearly significant for the OOC task (DHA more time, $P = 0.055$). Again, after adjusting for age as a covariate, the DHA group spent less total time interacting with the 1st cube ($P = 0.03$) and 2nd cube ($P = 0.0016$). For the OOC task, the DHA group spent more total time interacting with the cube after adjusting for age as a covariate ($P = 0.03$). The 1 year old birds spent significantly less time interacting with the cube prior to it being opened for the 1st cube opened ($P = 0.01$), 2nd cube opened ($P = 0.01$), 3rd cube opened ($P = 0.03$), red cube ($P = 0.006$), green cube ($P = 0.01$), and nearly significant for the yellow cube ($P = 0.05$). The 1 year old birds also spent significantly less time than the 3-4 year old birds interacting with the cube after it was opened in the OOC ($P < 0.0001$), EOOC ($P = 0.002$), ROOC ($P = 0.007$), and the 1st cube opened ($P = 0.0002$), 2nd cube opened ($P < 0.0001$), red cube ($P = 0.04$), and green cube ($P < 0.0001$) in the TCT task. An age effect was additionally observed for the total amount of training time spent interacting with a cube, with significantly less time for the 1 year olds in the OOC ($P = 0.002$), EOOC ($P < 0.0001$), ROOC ($P = 0.008$), and the 1st cube opened ($P < 0.0001$), 2nd cube opened ($P <$

0.0001), red cube ($P = 0.0007$), green cube ($P < 0.0001$), and yellow cube ($P < 0.0001$). The 3rd cube opened trended in the same direction, but was not significant ($P = 0.079$).

Discussion

The age of the birds turned out to be a highly significant factor in this study. It was expected that any age effects observed would be due to the 3-4 year old birds performing at a lower level than the 1 year olds. One reason for this hypothesis was that it is commonly believed that critical and optimal (also referred to as sensitive) learning periods occur in younger birds and thus be easier to train and learn faster than older birds (98, 99). Additionally, Yamamoto et al (100, 101) found that older rats (19.7 months) took 2 weeks to learn a shaping process that consisted of pressing a lever to receive a feed pellet 40 times in 20 minutes in a Skinner box under a bright light on a screen compared to 4-5 days in young rats (11 weeks). During a brightness-discrimination learning task in which the rats were only supposed to press the lever if the bright light was on, the aged rats had significantly fewer total responses than the young rats (100, 101). However, a significant diet effect despite age performance differences was observed with the rats fed a diet containing perilla oil (source of ALA) having a higher correct response ratio (higher success) than rats fed a safflower oil containing diet (100, 101).

Another reason for this expectation is because DHA has generally been shown to be incorporated into the brain and neural tissues faster and in greater amounts during growth of young mammals (102). Moriguchi and Salem (44) observed that 3rd generation ALA-deficient rats performed at the same level as ALA-adequate rats in a water maze 6

weeks after being switched to an ALA-adequate diet at 7 weeks of age (young adults), but not after only 2 weeks. Carrie and colleagues (103) observed reversal of learning and biochemical alterations in ALA-deficient mice when supplemented with DHA brain phospholipids from pigs at 8 months and tested at 17-18 months old (elderly). These findings suggest DHA can be incorporated into the brain of adult rats and mice, and may also apply to Monk parrots. The Monk parrots in our study were provided approximately 1.7 g DPA+DHA/kg feed/day for a total intake of about 196 mg DPA+DHA/day. While both the LALA and DHA diets used in this study had low dietary ALA (0.23 and 0.16 en%, respectively), this was likely not low enough or fed for a long enough period to produce deficiency in these birds. No signs of ALA or n-3 deficiency were observed in this study. Thus, it is possible that brain DHA levels were not reduced in the LALA group, thus any diet differences would likely be the result of increased DHA incorporation into the brain by the DHA group. However, it is unknown how much dietary n-3 is required to obtain brain DHA saturation, the rate of DHA incorporation, or DHA recycling in Monk parrots. The period of DHA supplementation prior to the ROOC task may have not been long enough to result in differences in brain DHA levels. Since DHA is incorporated into the hippocampus (42), it may be more beneficial to test memory of a task at some later time point while being maintained on the experimental diets rather than just initial learning.

However, decreased performance in the 3-4 year old birds was not the case and the opposite effect was observed in this study. The 1 year old birds consistently took longer to open the cubes and showed less time of interaction between initial contact and

opening the cube's lid. The 1 year old birds also consistently spent less time interacting with the cubes after the cubes were opened. The exact reasons for these age effects are unknown. While the one year old birds are considered adults, they may have not yet fully matured. Thus their motor skills might not be as well developed as the slightly older birds. It is also possible that the 1 year old birds were less feed motivated or more easily distracted than the 3-4 year old birds. Additionally, the 3-4 year old birds likely had more general experience being introduced to and manipulating novel enrichment items than the 1 year old birds. It may also be important that the 1 year old birds were obtained from feral populations prior to fledging whereas the 3-4 year old birds were obtained from breeders. Additionally, all birds were treated for zinc toxicity prior to the beginning of this study, with the 1 year old birds showing more symptoms. Due to the large difference in performance between the two age groups, future training studies should utilize birds of the same age or otherwise take into account the findings of the present study. Since the 1 year old Monk parrots had a poor performance rate in this study, avoiding their use in future training studies may improve completion rates which could aid in observing differences due to dietary components.

Overall, the DHA group took more time to make initial contact with and open the green cube during the TCT task, but interacted with the green cube more between these two time points. However, the DHA group spent less time interacting with and eating feed out of the green cube after the lid was opened. These results combined suggest the DHA group did not learn the TCT task as well as the LALA group by the end of the study despite there being no difference in percentage of successful sessions. The

decrease in interaction time with the green cube after the lid was opened seems to indicate that a feed reinforcement effect in the DHA group may not have been as large as in the LALA group.

All of the birds in this study were not entered into the TCT task, and even fewer completed this task. It is possible that the TCT task was too complicated for the birds. However, it is more likely the birds that made it beyond the ROOC task were not provided an adequate number of sessions in order to successfully complete the TCT task. It is also possible that the birds experienced training fatigue, especially towards the end of the study. If so, it may be beneficial to provide the birds with more training-free days in a week. The large training cage size combined with being located in a room with the normally housed Monk parrots could have provided additional distractions when they otherwise may have been completing the expected tasks. Another possible distraction may have occurred when a second bird was placed into its training cage shortly after the first bird was placed into its training cage. However, the total time to place the second bird averaged only 60.7 ± 15.2 seconds. The general shape and sharpness of each bird's beak could have also led to variations in performance, with some shapes being more beneficial for opening a cube than others.

Most parrots have been observed to be left footed, meaning the bird perches on their right foot and holds feed in the left foot (104). Based on observations of which foot the bird ate their feed with, one bird in this study was predominantly right footed and the remaining 10 were left footed. Brown and Magat (105) found a significant positive correlation for foot preference and eye preference in 11 parrot species (out of 16

studied), meaning foot preference was a means to bring the object closer to the preferred eye. Eye preference can be linked to brain lateralization, which has been demonstrated in chickens (106). Since each hemisphere of the brain is responsible for specific types of information (106), variation in footedness may be an indicator of differences in brain usage and thus possibly affect learning ability/performance. Right footed African Grey and Amazon parrots had a significantly larger lexicon than their left footed counterparts (107). Because the Monk parrots used in this study were predominantly left footed, it is unlikely that foot preference affected the results.

This study appeared to have ample power with the small sample size for determining age effects. However, accounting for age resulted in lowering the sample size in the diet groups, so it is likely the study was no longer adequately powered to analyze for diet effects. It would be beneficial in future studies to have a larger sample size with all birds the same age in order to determine whether dietary DHA actually affects the learning ability of Monk parrots.

CHAPTER V

GROWTH CURVES AND THEIR IMPLICATIONS IN HAND-REARING MONK PARROTS

Introduction

The maintenance energy requirements of most parrot species have not been fully established. Most research on energy requirements in avian species has focused on poultry, especially chickens, as well as budgerigars (108). Information on poultry may have limited applicability to parrot species because poultry species are precocial whereas parrots are altricial. Poultry species have also been studied in order to maximize the growth rate for meat production, which is undesirable for pets and conservation purposes. While there is information on budgerigars, these birds are one of the smallest parrot species and may thus differ in energy requirements in terms of body weight from larger parrot species. The growing energy requirements of parrots has been studied even less, although it could possibly be estimated from the adult maintenance energy requirements as done with dogs and domestic cats (109).

Hand-rearing baby birds is commonly performed by pet bird breeders, who have established individualized methods over years of experience and previous success. These techniques, however, do not ensure the birds will achieve optimal health as adults. While individualized, established methods result in the birds surviving to adulthood, they may additionally result in excess body fat stores as weanlings. Many chronic, progressive inflammatory diseases, including atherosclerosis, are linked to excess body fat. To help

improve bird health, it is important to determine the energy requirements of adult and growing birds of many species and to ensure public access to this information. In this study, we compared the growth curves of hand-reared Monk parrots to those of parent-raised birds in order to determine where differences might exist and to provide a better estimate of their energy requirements during post-hatching development.

Materials and Methods

2010 hand-raised birds

A number of feral Monk parrots ranging in age from embryos to nestlings were donated to the Schubot Exotic Bird Health Center at Texas A&M University during the summer of 2010. Recommendations and methods for hand-rearing parrot chicks were sought from established breeders and researchers (110) as well as from feed manufacturers. Because protocols for hand-rearing Monk parrots specifically were not established, feedings based on general guidelines were employed. Briefly, this consisted of feeding commercial formulas either by tube or syringe until the crop appeared full. Feed volumes as a percentage of body weight known to be safe and considered adequate for baby birds at varying ages were employed as guidelines (111). The crop was kept full during the day. However, crops were checked to ensure complete emptying overnight, unless the bird was under 10 days of age.

2011 hand-raised birds

Additional feral Monk parrots ranging in age from embryos to early fledglings were donated to the Schubot Exotic Bird Health Center at Texas A&M University during late Spring and Summer, 2011. In an attempt to prevent potential health issues due to

delayed growth followed by catch-up growth experienced by the 2010 hand-raised bird group, energy requirements of growing Monk parrots were more closely estimated in order to establish a protocol for hand-rearing baby birds during 2011. The feeding protocol is shown in Table 17. The energy requirements of growing Monk parrots were estimated by doubling a previously published existence energy requirement equation of non-passerine birds (0.5404*W^0.7545, W = g) (112) and mammals (110*W^0.75, W = kg) (109). A similar strategy has been recommended for small mammals including growing canine species (109). The metabolizable energy content of selected commercially available hand-feeding formulas was estimated by calculation from the guaranteed analysis (Appendix D). The metabolizable energy content was used in conjunction with known safe volumes per feeding (percent of body weight) (110) to determine the number of feedings required to meet the estimated daily energy requirements of the growing Monk parrots. The desired feed dilutions for providing necessary water in addition to ease of feeding were also considered in the calculations. The birds were tube or syringe fed according to body weight following the protocol in Table 17. The ages of the birds were estimated from their individual body weights on the first full day of Schubot Exotic Bird Health Center possession using data from the 2010 hand-reared birds and parent-reared Monk parrots (113). Because there were a large number of birds, they were divided into four smaller groups consisting of 3-8 birds based on weight and stage of development at time of acquisition.

Table 17. Feeding protocol for 2011 hand-reared Monk parrots.

Age in days	Expected weight (grams)	Volume per feeding	Mix of parts formula to parts water F:W (kcals/ml)	Feeding Times
0-4	5-9	0.3-0.7 ml	1 part formula A:3 parts water (1.0 kcal/ml)	3, 6, 8, 10, 12, 14, 16, 18, 20, 22, midnight
5-8	10-15	0.5-1 ml	1 part formula A: 2 parts water (1.3 kcal/ml)	6, 8, 12, 14, 16, 18, 20, 22, midnight
9-21	20-50	2-5 ml	1 part formula B: 3 parts water (0.82 kcal/ml)	6, 9, 12, 15, 19, 22
22-40	60-80	6-8 ml	1 part formula B: 2 parts water (1.1 kcal/ml)	6, 10, 14, 18, (22)
41-60	90-120	6-10 ml	1 part formula B: 2 parts water (1.1 kcal/ml)	6, 10, 14, (20) offer weaning foods

Commercially available diets were used for this protocol. Feed dilution and number of feedings were calculated to meet the estimated energy requirements of the growing Monk parrots using the estimated calories of the chosen commercial diets. Volume per feeding was estimated from percent of body weight that can be safely fed to baby birds (110). Times are listed in 24-hour clock format.

Results

2010 hand-raised birds

Realizing the potential opportunity for hand-rearing additional birds the following year, growth curves for this initial group were compared to a previously published growth curve for parent-reared Monk parrots (Figure 1) (113). Data was thus obtained on 7 Monk parrots during growth, however only 5 survived past fledging. Figure 13 illustrates a delay in growth of the hand raised birds occurring up to approximately day 28 post-hatch. The birds then experienced a rapid period of catch-up growth. The birds continued to grow leading to significantly higher peak body weights prior to fledging as compared to the parent-reared birds (Table 18, 121.2 g hand-raised versus 97.7 g parent-raised) (113). The hand-reared birds also experienced slightly delayed fledging and weaning times. In addition, stepwise weight loss patterns were seen near the time of fledging in the hand-reared birds compared to continuous weight loss in the parent-reared birds (113).

2011 hand-raised birds

Data was obtained from 18 hand-reared Monk parrots. The growth curves for the four groups were compared to the 2010 hand-reared and parent-reared Monk parrots (Figure 14). Two of the groups [Black Dot (n = 6) and Green Dot (n = 3)] closely followed the parent-reared birds data while two groups [Pink Dot (n = 6) and Red Dot (n = 3)] diverged to a slower growth rate around day 20. The growth curve for the average of all chicks hand-reared in 2011 closely followed that of the parent-reared chicks up to approximately day 30 post-hatching (Figure 15).

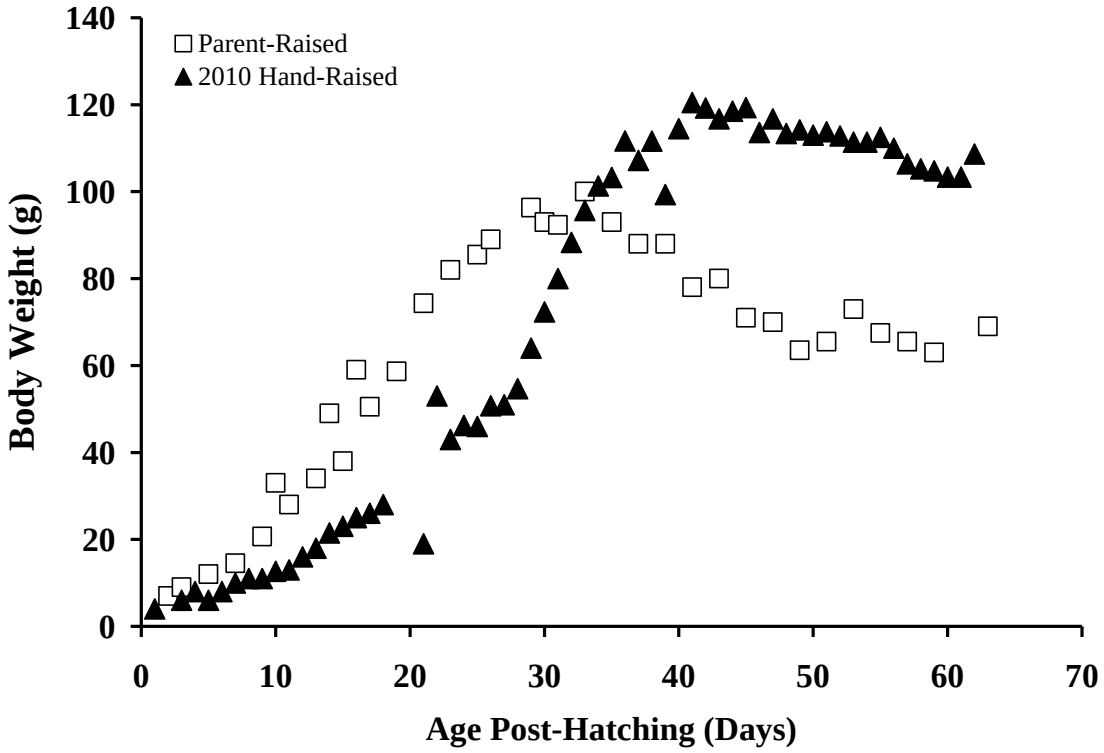

Figure 13. Growth curves for Monk parrots hand-raised by Schubot Exotic Bird Health Center in 2010 and parent-raised as reported by Caccamise and Alexandro (113).

Table 18. Growth rate indicators in Monk parrots hand-raised at Schubot Exotic Bird Health Center compared to parent-raised Monk parrots (113).

	Black Dot	Green Dot	Pink Dot	Red Dot	Hand-Raised 2010	Parent-Raised
Number of Observations	n=6	n=3	n=6	n=3	n=7	n=3
Peak Weight Pre-Fledging (g)*	$113.0^{a,b}$	125.0^{a}	$112.0^{a,b}$	$105.7^{a,b}$	121.2^{a}	97.7^{b}
Age at Peak Weight (days)**	34.3^{c}	$34.0^{b,c}$	$40.8^{a,b}$	45.3^{a}	42.8^{a}	29.3^{c}

Letters that differ across rows are significantly different ($P < 0.05$). Colored dots are the sub-groups of birds hand-raised in 2011. * $P = 0.016$, ** $P < 0.0001$.

The peak body weight prior to fledging and the age to reach peak body weight was compared to the 2010 hand-reared birds and the parent-reared birds (Table 18) (113). There was no significant difference in peak body weight prior to fledging between any of the sub-groups hand-raised in 2011 and the chicks hand-raised in 2010. The chicks hand-reared in 2010 and the Green Dot group from 2011 reached significantly higher peak body weights than the parent-reared birds. The age to reach peak body weight was not significantly different between the Pink Dot group, Red Dot group and the 2010 hand-reared birds. However, these three groups did take significantly longer than the parent-reared birds and the Black and Green Dot groups to reach their peak body weights. There was no significant difference in age at peak body weight between the parent-raised chicks and the Black and Green Dot groups.

Discussion

2010 hand-raised birds

The delayed growth experienced in the 2010 hand-raised birds may be associated with delayed motor development. Furthermore, the period of rapid weight increase may also lead to other health issues later in life. For example, excess weight gain prior to fledging in addition to starting their adult life weighing more than parent-reared birds (113), could potentially increase their risk of developing atherosclerosis and other chronic, progressive inflammatory diseases as adults. Ricklefs (114) reported that weight loss experienced by nestlings immediately prior to fledging is associated with decreased water content while total lean dry mass and lipid content remained constant. Thus, excess lipid accumulation during growth is not significantly lost prior to adulthood.

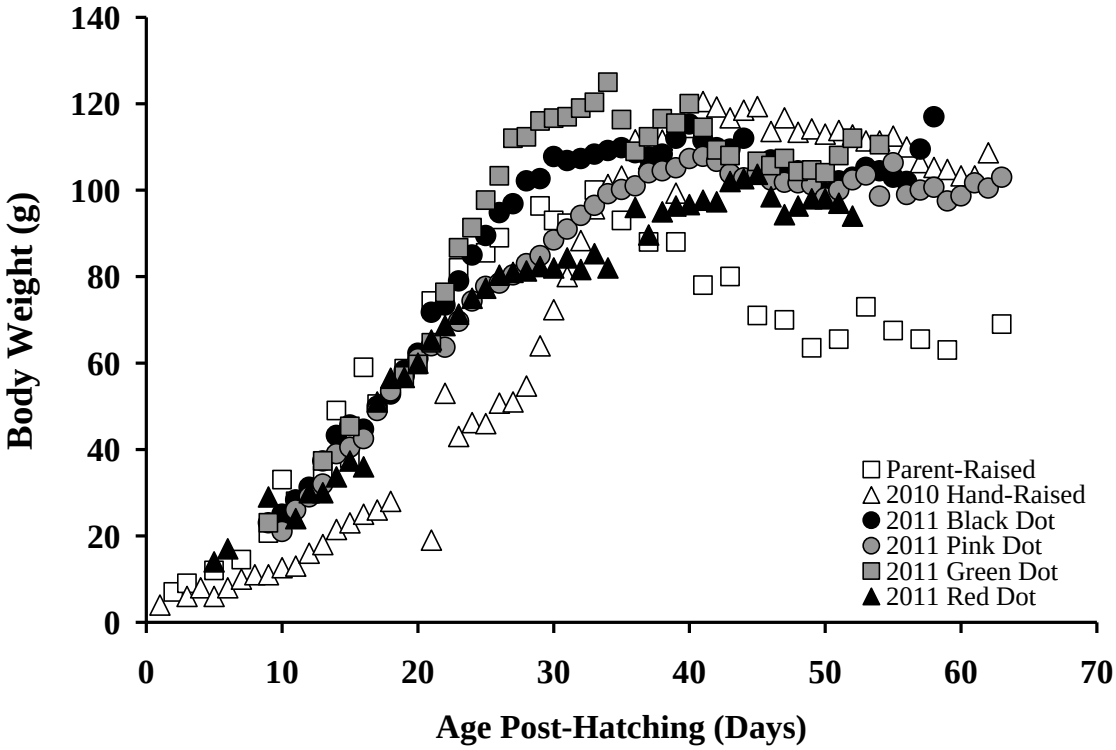

Figure 14. Average growth curves for Monk parrot sub-groups hand-raised by Schubot Exotic Bird Health Center in 2011 by group compared to previous data.
The birds hand-reared in 2011 were split into four groups, Black Dot, Pink Dot, Green Dot, and Red Dot for ease of handling. The parent-reared data is from Caccamise and Alexandro (113).

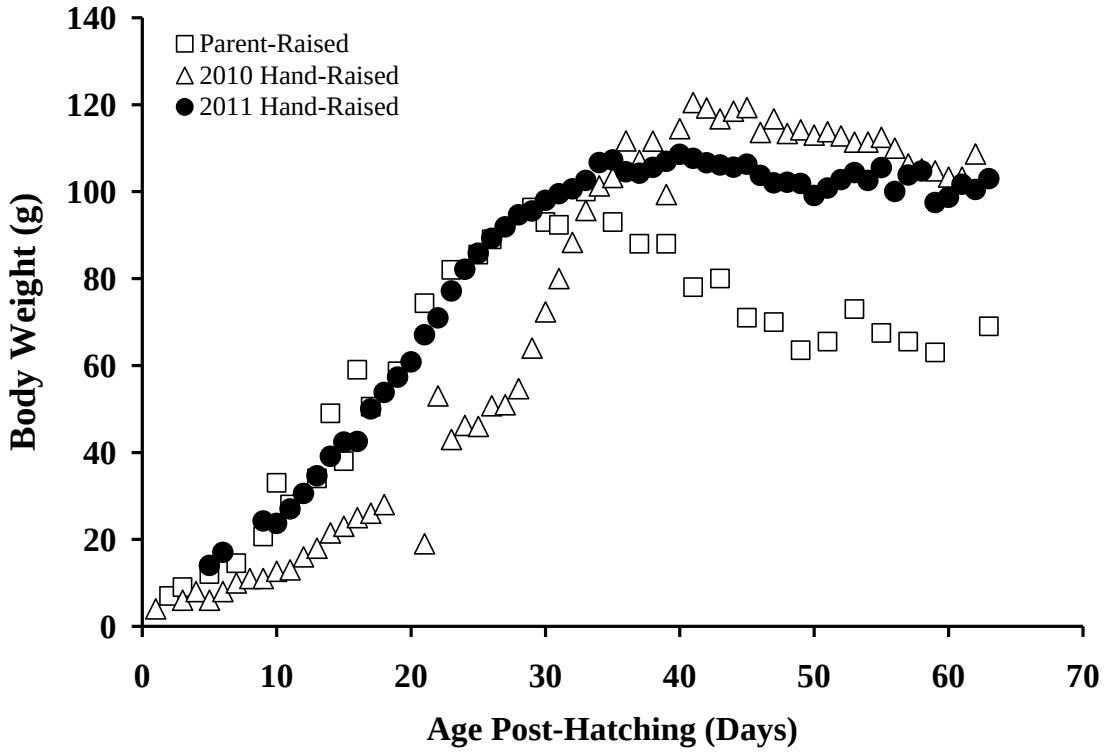

Figure 15. Average growth curves for Monk parrots hand-raised by Schubot Exotic Bird Health Center in 2011 compared to previous data.

Obesity in infants has also recently been associated with delayed motor development, and may have a similar effect in Monk parrots if they become overweight when young (115). Due to the multitude of potential health issues that may result from excessive growth rate and/or excessive weight gain when young, research should focus on the energy requirements and methods of hand-rearing Monk parrots in order to prevent these conditions from developing.

2011 hand-raised birds

Analysis of the energy intake of the four sub-groups hand-raised in 2011 revealed the Pink Dot and Red Dot birds were fed according to the protocol shown in Table 2. The Black Dot and Green Dot groups received more energy per day than the protocol called for beginning around day 20. Since the Black Dot and Green Dot groups closely followed the growth curve of the parent-reared Monk parents up to 30 days of age, it can be concluded that the 2-fold increase of the existence energy equation for non-passerines originally estimated was lower than their actual energy requirements.

To better understand why the equations used underestimated the energy requirements of growing Monk parrots, the energy intake of adult Monk parrots maintaining weight and housed at the Schubot Exotic Bird Health Center (figured from data collected from studies explained in Chapters II and III) were compared to the non-passerine existence energy requirement equation (112). Adult Monk parrots maintaining weight consumed 1.87 times more energy than estimated by the Kendeigh (112) equation. A comparison was then made between the average energy intake of the birds hand-reared in 2011 to the modified equation for adult Monk parrot maintenance (1.87*0.5404*W^0.7545 or 1.02*W^0.7545, W = g) (Figure 16). On first observation, it appeared that the hand-reared chicks consumed 1.4 times the adult Monk parrot maintenance energy requirement (1.43*W^0.7545, W = g). However, upon further inspection three different periods of energy intake were noted in the 2011 hand-reared chicks. Between day 0 and 18 of life, the chicks consumed on average 1.02*W^0.7545

(W = g), the same as the adult Monk parrot maintenance energy requirement. During day 18 to 23 of growth, the hand-reared chicks consumed 1.43*W^0.7545 (W = g) or 1.4 times adult Monk parrot maintenance energy requirements on average. The possibility exists that this increase in energy needs corresponds to the production of adult feathers. Observations by Caccamise (116) puts the emergence of primary feathers at day 18 with good delineation by day 24. The head and wing feathers were also fully emerged by day 24, but many contour feathers on the body were not fully completed until day 37 (116). After day 23, it was observed that the chicks total energy intake per day remained relatively constant, but the chicks continued to increase body weight. This suggests a decrease in the energy requirements of the chicks occurs after 23 days of life. Work in chickens by Ricklefs suggests that this decrease could be due to a decrease in the chick's basal metabolic rate (117).

The delay in age to reach peak body weight observed for the Pink Dot, Red Dot, and 2010 hand-reared birds is likely representative of a slower growth rate for these birds. It is also important to note that although the Black Dot group grew at a faster rate than the Pink Dot group, their peak body weight prior to fledging was not different. Thus growth rate is not necessarily indicative of the peak body weight that a bird will attain prior to fledging.

Ricklefs (118) determined a graphical method of determining the K constant for use in comparing growth rates between avian species. Using this technique, Caccamise and Alexandro (113) have determined the K constant for parent-raised Monk parrots to be K = 0.1624. Following the same method, the K constant for the average of the chicks

hand-reared in 2011 was determined to be K = 0.1676, and similar to the parent-reared chicks.

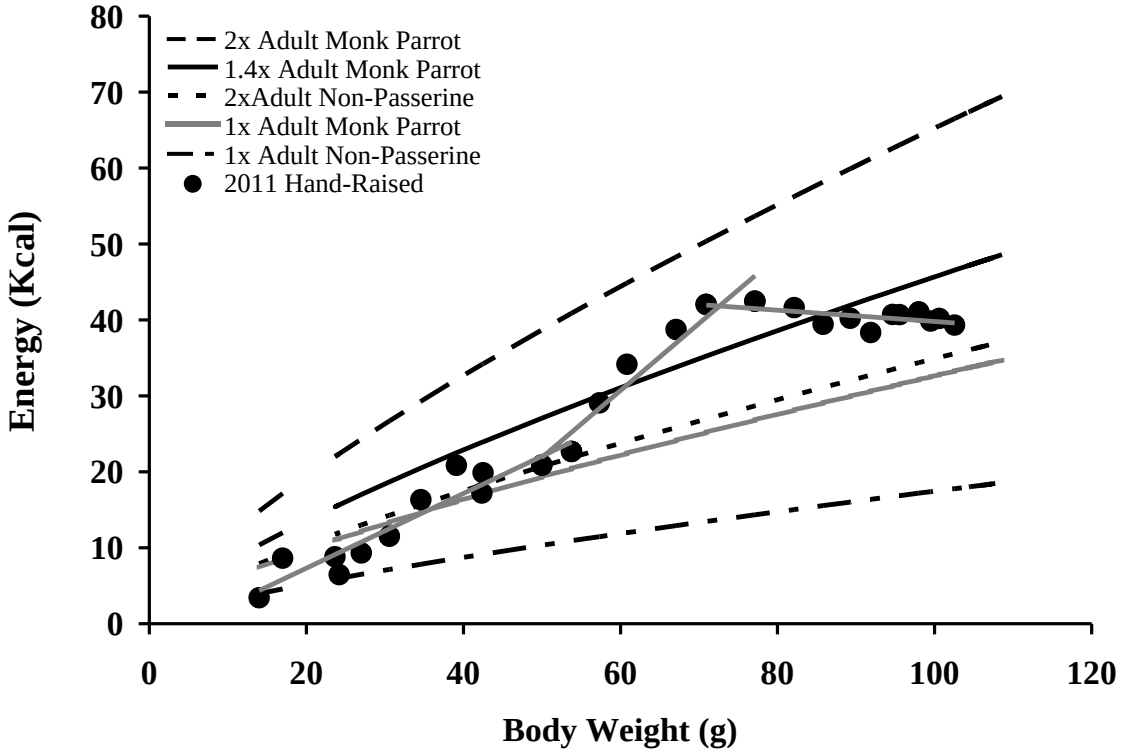

Figure 16. Average energy intake of the average of all birds hand-raised in 2011 compared to energy equations.
Birds hand-raised in 2011 (filled circles) compared to one and two times the non-passerine maintenance energy equation (112) used in the protocol shown in Table 2, the modified equation representing adult Monk parrot maintenance energy requirements (1.87*0.5404*W^0.7545, W = g), 1.4 times the adult Monk parrot maintenance energy requirements, and 2 times the adult Monk parrot maintenance energy requirements. The gray lines show the changes in energy needs as the Monk parrots grew.

CHAPTER VI

CONCLUSION

Monk parrots were able to convert α-linolenic acid (ALA) to both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Thus unlike humans, Monk parrots do not have to consume DHA in the diet. They were further able to retro-convert DHA to EPA and docosapentaenoic acid. This ability to retro-convert DHA suggests that Monk parrots can still receive the beneficial less pro-inflammatory effects of EPA, without having to consume EPA or ALA in the diet. A high ALA diet also resulted in shifting the peak density of high-density lipoproteins compared to a high linoleic acid diet. The biological consequences of this density shift are unknown at this time. A dose response was observed to increasing dietary ALA with a shift in accumulation patterns occurring around a dietary inclusion level of 3.2 en% ALA. Thus, diets containing more than 3.2 en% ALA may result in exceeding thresholds for many enzymes and was observed to increase accumulation of most n-3 PUFA in the plasma phospholipid. This increased accumulation of n-3 PUFA could possibly provide additional protective effects against atherosclerosis than diets containing less than 3.2 en% ALA. Higher plasma phospholipid accumulations of DHA occurred when ALA was replaced by DHA in the diet. Thus providing DHA in the diet may provide more beneficial and protective effects for preventing the development of atherosclerosis than an equivalent level of ALA.

Many risk factors for atherosclerosis have been determined in humans and other mammals. However, these risk factors may not hold for Monk parrots and possibly other

birds prone to developing atherosclerosis. When feeding a high n-3 PUFA diet the same alterations in lipoproteins and lipid metabolism as previously observed in humans and other mammals did not occur. Monk parrots, similar to canines, have HDL as their major lipoprotein fraction. In dogs, this is considered one of the reasons they are resistant to developing atherosclerosis. However, Monk parrots are prone to developing atherosclerosis. Additional support for why dogs are resistant to atherosclerosis is that when fed a high fat diet, they can maintain their relative percent LDL and VLDL and only increase the relative percent of the HDL fraction as total cholesterol increased. When the Monk parrots were subjected to a comparably high fat diet in these studies, the relative percent of ß actually decreased as total cholesterol increased and no changes in pre-ß or α were observed. In most mammals, feeding increased levels of n-3 PUFA decrease total cholesterol and triacylglycerols. However, no effect on total cholesterol or triacylglycerols were observed as dietary ALA was increased in this study. Additionally, in humans, a "longevity pattern" predictive of low risk of developing atherosclerosis has emerged which is characterized by a decrease in HDL_{3c} and HDL_{3b} and an increase in HDL_{2b}. However, in the Monk parrots fed a high ALA diet, the opposite shifts occurred with HDL_{3c} increasing, no change in HDL_{3b}, and HDL_{2b} decreasing. These species comparisons to the effect of feeding dietary n-3 PUFA on atherosclerotic risk factors may help determine better risk factors and possibly the etiology for atherosclerosis. Though it is possible that the etiology for atherosclerosis differs between mammals and avians.

An effect of DHA on the learning ability of Monk parrots was not able to be concluded. However, it was observed that 1 year old Monk parrots performed significantly worse than 3-4 year old Monk parrots. The reason for this difference is not known at this time. Future research on dietary effects on learning ability in Monk parrots and possibly other avian species should utilize birds of the same age and preferably older than 1 year.

It is important to consult growth curves while hand-rearing baby birds in order to optimize their health and prevent large changes in their growth rate. Due to the potential health implications from delayed development followed by excessive growth, more information about the growth requirements and caloric needs of Monk parrots are needed, especially at the time around fledging. The similar K constant found for the birds hand-reared in 2011 with those parent-reared suggests that the birds hand-reared in 2011 grew at an acceptable rate. This also suggests that the energy consumed by the 2011 hand-reared birds is the same amount the parents provided their chicks. Thus, our protocol (1.02*Wg^(0.7545) provided adequate energy through day 18 (~54 g). Between d18-23 Monk parrot chicks should be provided 1.4*Wg^(0.7545). Additionally, more specific and accurate information on hand-rearing healthy birds by species needs to be available to the general population in an easily accessible manner.

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APPENDIX A

Equation used to correct feed intake for the desiccation standard.

First, the desiccation correction factor (DCF) is determined.

DCF = (desiccation in – desiccation out) / desiccation in

Then, the feed out (the sum of feed removed, feed dropped, and feed in water dish in this case) is adjusted by the DCF.

feed out = feed removed + feed dropped + feed in water dish

adjusted feed out = feed out * DCF

Finally, the adjusted feed out is subtracted from the feed in (offered) to provide the amount of feed consumed.

feed consumed = feed in – adjusted feed out

APPENDIX B

Equations used to determine formaldehyde concentration and catalase activity.

The average absorbance of the 0 μM formaldehyde standard was subtracted from the average absorbance of each standard and sample. The corrected absorbance of each standard was plotted as a function of the standards final formaldehyde concentration (μM). Linear regression of the new standard curve provided an equation that could be used to determine the formaldehyde concentration of each sample.

$$\text{formaldehyde } (\mu\text{M}) = \left[\frac{\text{average sample absorbance} - (\text{y - intercept})}{\text{slope}} \right] \times \frac{0.17 \text{ ml}}{0.02 \text{ ml}}$$

Catalase activity was then calculated from formaldehyde concentration.

$$\text{catalase activity (nmol/min/ml)} = \frac{\text{F } (\mu\text{M}) \text{ of sample}}{20 \text{ minutes}} \times \text{sample dilution}$$

APPENDIX C

Equation used to determine superoxide dismutase activity.

The average absorbance of the 0 final SOD activity (U/mL) standard was divided by the average sample and standard absorbances $\left(\frac{\text{average absorbance sample1}}{\text{average absorbance 0 U/mL standard}}\right)$ to give the linearized rate. The linearized SOD rates were then plotted as a function of final SOD activity (U/mL). The linear regression of the new standard curve provided an equation that was used to determine superoxide dismutase activity.

$$\text{SOD (U/mL)} = \left[\left(\frac{\text{sample linearized rate} - (\text{y - intercept})}{\text{slope}}\right) \times \frac{0.23\,\text{mL}}{0.01\,\text{mL}}\right] \times \text{sample dilution}$$

APPENDIX D

Equation used to determine metabolizable energy of commercially available hand-feeding formulas.

Metabolizable energy of diet (kcal/100g diet) = energy from protein (kcal/100g diet) + energy from fat (kcal/100g diet) + energy from carbohydrates (kcal/100g diet)

Energy from protein (kcal/100g diet) = g crude protein/100g diet x 3.5 kcal/100g protein

Energy from fat (kcal/100g diet) = g crude fat/100g diet x 8.5 kcal/100g fat

Energy from carbohydrates (kcal/100g diet) = g carbohydrates/100g diet x 3.5 kcal/100g carbohydrates

To calculate carbohydrates from guaranteed analysis:

carbohydrates (g/100g diet) = 100g diet – g protein – g fat – g fiber – g ash – g moisture

VITA

Christina A Petzinger received her Bachelor of Science degree in animal science from Berry College in Rome, GA in 2009. She entered the Nutrition program at Texas A&M University in August 2009 and received her Doctor of Philosophy degree in Nutrition in May 2012. Her research interests include comparative animal nutrition, especially exotic species, and lipid metabolism.

Christina A Petzinger may be reached at Department of Small Animal Clinical Sciences, Texas A&M University, MS 4474, College Station, TX 77845-4474. Her email is christina.petzinger@vikings.berry.edu.